Bioremediation of modelled petroleum-contaminated soils of the Niger Delta and the impact of zeolite augmentation

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ABSTRACT

The bioremediation of modelled petroleum oil-contaminated soils of the Niger Delta by a mixed culture of three hydrocarbon-degrading bacteria, *Acinetobacter sp, Rhodococcus sp* and *Pseudomonas sp,* was investigated. These bacterial strains were selected based on criteria that they were able to utilize hydrocarbons (hexadecane and sodium benzoate) as the sole source of carbon and energy and were able to show significant growth in crude oil at an optimum temperature of 30^{o}C. For maximal bacterial growth and degradation effective aeration and agitation was required, thus the choice of the shake flask method over the bioscreen growth analyzer for this investigation. The influence of hexadecane concentrations (0.5%, 1.0% and 2.0%) on the bacterial isolates was investigated and it revealed that the *Rhodococcus sp* because of its different metabolic pathway showed a more rapid growth on hexadecane concentrations as compared *Pseudomonas sp and Acinetobacter sp*. Amongst the bacterial isolates, *Pseudomonas sp* exhibited a more rapid growth on 0.5% sodium benzoate while the two others showed minimal growth. *Pseudomonas sp Rhodococcus sp* and *Acinetobacter sp* showed a synergistic association when grown on basal salt medium supplemented with 1.0% w/v petroleum crude oil.

The influences of a zeolite (clinoptilolite), soil structure and particle size on biodegradation of crude oil in modelled silt-clay and sandy soil of Niger Delta was investigated. Soils from the Hilton site, East-Shropshire, United Kingdom were used for the Niger Delta soils formulation. Geochemical properties of the soil samples from the x-ray fluorescence showed major elements are sodium, magnesium, aluminum, potassium, iron with silicon having high percentage, while x-ray diffraction analysis revealed minerals such as quartz, kaolinite, illite and smectite, which are similar to those of the Niger Delta.

The preliminary investigation showed a more rapid and greater extent of apparent oil removal with the addition of both bacterial consortium and clinoptilolite on soil amendment experiments at 30^{o}C for a period of 30 days. There was 79% oil removal by the bacterial consortium in the soil amended with clinoptilolite as compared to 67% in the case of the amended soils without clinoptilolite. Although the addition of both bacterial consortium and clinoptilolite enhanced the removal of the crude oil, however the effect of clinoptilolite may be one of abiotic removal. The soil structure investigation without clinoptilolite augmentation showed that oil removal in the silt-clay soil was significantly greater than that of the sandy

soil after 30 days period ($p < 0.0001$). There was 72.7% ± 0.8% oil removal by the bacterial consortium in the silt-clay soil as compared to 55.6% ± 0.7% in the case of the sandy soil. However, there was 79.1% ± 0.4% oil removal by the bacterial consortium in the silt-clay soil amended with clinoptilolite as compared to 67.3% ± 0.8% in the case of the amended sandy soils with clinoptilolite. Gas chromatographic profile showed appreciable reductions in hydrocarbon, the rate of which depended upon the particular hydrocarbon. Quantitative analysis of residual oil extract from the silt-clay and sandy soil amended with and without zeolite showed a high rate of degradation for lighter hydrocarbon components (C_{10}– C_{18}) compared to the heavier ones (C_{24} – C_{28}) by the bacterial consortium. Hydrocarbon components (C_{10}– C_{18}) from both silty-clay and sandy soils amended with zeolite were degraded by the bacterial consortium to 92.1% - 57.7% and 74% - 43.7% respectively, while the soils without zeolite showed degradation rate of 80.4% - 44.8% (silt-clay) and 69.4% - 42.8% (sandy). Hydrocarbon components (C_{24}– C_{28}) from both soils showed an apparent low rate of degradation. The results of this study indicate that the application of the bacterial consortium and clinoptilolite lead to greater rates of biodegradation in the clay soil then in the sandy soil. Studies showed that nutrient addition and aeration both affected the rate of hydrocarbon utilization. The postulated application of selected bacteria in the bioaugumentation of oil contaminated environment in the Niger Delta region was discussed.

DEDICATION

This work is dedicated to the Almighty God, and to the evergreen memory of my humble father-late Joseph A. Ogala, my dear Mum- Mrs Mary Ogala, my beloved wife – Mrs Louise Annemarie Joseph and my family for their love, prayers, encouragement and commitment to my academic pursuit.

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LIST OF ABREVIATIONS

ANOVA	Analysis of Variance
BGA	Bioscreen growth analyzer
BSM	Basal salt medium
BSMA	Basal salt medium agar
BTEX	Benzene Toluene Ethylbenzene and Xylene
Cfu	Colony counting units
CSTR	Continuously stirred tank bioreactor
DF	Dilution factor
DGGE	Denaturing gradient gel electrophoresis
GC	Gas chromatography
HEX	Hexadecane
HA	Hexadecane agar
OD	Optical density
SB	Sodium benzoate
SD	Standard deviation
SEM	Scanning electronic microscopy
SFM	Shake flask method
TPH	Total petroleum hydrocarbon
TSA	Trytone soy agar
TVC	Total viable count
UWCC	University of Wolverhampton culture collection
PAH	Poly-aromatic hydrocarbon
PCO	Petroleum crude oil
PH	Petroleum hydrocarbon
rpm	Revolutions per minute
w/v	Weight per volume
XRF	X-ray fluorescence
XRD	X-ray diffraction

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

1.1 INTRODUCTION

Oil pollution is considered a global concern to both humans and the environment. In England and Wales alone, 12% of all serious contamination incidents in 2007 were hydrocarbon related (Environment, 2005). In the United States of America, more than 1,200 petroleum-hydrocarbon contaminated sites have been remediated, and about additional 32,000 sites were reported with potential contamination problems (Liu *et al.,* 2008).

In Nigeria, there is an average of 300 oil spills every year. Environmentalists consider the Niger Delta region of the country as one of the world's most polluted regions (Sojinu *et al.*, 2010). Most of the terrestrial oil spills in this region occur in areas with difficult terrain, which hinders *in situ* biodegradation studies. When research is conducted, outcomes are delayed after accidental spills because owners of land devastated by the spills must be remunerated before studies are allowed on such sites. Therefore, data generated from such studies cannot be reliably used to immediately explain the impact of oil spills in Nigeria (Ijah and Antai, 2003). In addition, the Niger Delta region does not have effective regulatory laws and cannot afford the advance techno-centric approach to pollution problems. This concern influenced the need to research and to develop an effective but affordable bioremediation strategy to assist in solving oil pollution concern using commonly available microbial cultures and zeolite.

1.2 Bioremediation of hydrocarbon

The clean up of soil and the environment of petroleum hydrocarbon contaminant has remained a high priority (ASTM, 1995). The conventional remediation practice is to excavate

contaminated soil and place in a landfill site for natural attenuation. Biostimulation and bioaugmentation are the two major approaches to bioremediation (Korda *et al.,* 1997). These approaches are also considered to enhance microbial activity in oil-contaminated piles (Rahman *et al.,* 2000; El Fantroussi and Agathos 2005). Consortia of several bacteria have been reported to show an improved degradation of complex oil hydrocarbon as compared with single strains of bacteria (Rahman *et al.*, 1995).

Megharaj *et al.* (2011) reported that many intensive chemical substances are used in the built environment, e.g. petroleum oil, aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), BTEX (benzene, toluene, ethylbenzene and xylenes), pesticides, organic solvents and heavy metals all contribute to environmental pollution. Bioaugmentation technology has mostly been used for the degradation of pure compounds. Mineralization of a large concentration of the hydrocarbon phenanthrene has been reported (Schwartz and Scow, 2001).

MacNaughton *et al.* (1999) used molecular ecological approaches to analyze bacterial populations that occur in petroleum-contaminated marine environments and observed that *proteobacteria* appeared in the denaturing gradient gel electrophoresis (DGGE) fingerprints obtained for oiled plots but not in those for un-oiled plots. Biostimulation increase the indigenous concentration of microorganisms and speed up the biodegradation rates by improving nutrients and the bioavailability of the contaminants (Si-Zhong *et al.*, 2009). The commonly used technique is to introduce inorganic nutrients or oleophilic fertilizers onto the site (Allard and Neilson, 1997).

1.3 Bioremediation strategies

Bioremediation strategies are classified as *in situ* or *ex situ* (Aggarwal *et al*., 1990). Bioremediation processes are employed and effective in the treatment of diesel oil

contaminated soil in temperate climates (Ghazali *et al*., 2004; Riffaldi *et al*., 2006). The rate at which hydrocarbons are remediated differs between the *in situ* and *ex situ* bioremediation methods (Jorgensen, 2007)

1.3.1. *In situ* bioremediation processes

In situ bioremediation has been effective for the treatment of groundwater contaminated with mixtures of chlorinated solvents such as trichloroethylene, pentachlorophenol, carbon tetrachloride (Dyer *et al.,* 2003; Klecka *et al.,* 1998,); contaminants such as fuel and gasoline (Curtis and Lammey, 1998; Bhupathiraju *et al.,* 2002), oil wastes (Guerin, 2000) and coal tar contaminated sites (Durant *et al.,* 1997). This process involves treating the contaminant at the site of the contamination (Aggarwal *et al.,* 1990) and is divided into bioattenuation, biostimulation and bioaugmentation. *In situ* bioremediation can be also considered as natural and engineered bioremediation (Farhadian *et al.,* 2008)

1.3.1.1 Natural bioremediation

Kao and Prosser, (2001) reported that the main method of monoaromatic degradation is natural bioremediation and that 90% of BTEX removal by this approach can be attributed to intrinsic biodegradation processes. Natural attenuation is often limited by nutrient availability and the concentration of the electron acceptor (Hunkeler *et al.*, 2002). It is more cost effective than engineered condition (Andreoni and Gianfreda, 2007) and removes or decreases the concentration of organic pollutants from many contaminated sites (Roanling and Verseveld, 2002). There have been conflicting conclusions for the effectiveness of bioaugmentation and biostimulation; Van Hamme *et al.* (2003) concluded that addition of a commercial microbial culture to contaminated soil was not more advantageous than adding nutrients, but a recent study by Liu *et al.* (2008) reported contrary results, where biostimulation produced a more significant biodegradation rate. Natural attenuation proved to be more effective than

biostimulation and the number of diesel-degrading organism and the heterotrophic population was not influenced by the treatment (Bento *et al*., 2005). Some investigations of BTEX removal from contaminated site through natural *in situ* bioremediation are summarised in Table 1.1.

Table 1.1: Published studies of investigation of BTEX removal from contaminated site via natural in situ bioremediation

Contaminant	Electron acceptor (s)	Result (s)	Reference
Benzene	Sulphate	Benzene degradation occurred in sulphate-reducing condition, data showed an effective natural attenuation results and benzene was remediated with sulphate as electron acceptor.	Vogt *et al.,* 2007
BTEX and PAH (Tar oil)	Nitrate, ferric, sulphate, methanogenic and oxygen aerobic condition	BTEX and PAH were degraded; benzene and phenanthrene were degraded in the presence of Fe (III). The microcosm investigated utilized ethylbenzene and naphthalene under sulphate and Fe (III) reducing environment.	Schulze and Tiehm, 2004
BTEX (Petroleum)	Fe(III) and methanogenic condition	Results from the contaminated site with petroleum hydrocarbon shows that toluene and xylenes were degraded under methanogenic condition while benzene and ethylbenzene grew as Fe (III) supplies decreased.	Reinhard *et al.,* 2005
BTEX (Petroleum)	Aerobic condition	Results reviewed that approximately 88% of the BTEX removal was as a result of natural biodegradation processes. 87% of the total benzene, toluene, ethylbenzene and xylene (BTEX) isomers removal was observed via natural attenuation.	Kao and Prosser, 2001
BTEX (gasoline)	Aerobic and methanogenic condition	Assimilation potential of dissolved oxygen, ferrous iron and methane distributions when compared to BTEX concentrations reviewed ground water ability to degrade all dissolved BTEX	Kampbell *et al.,* 1996

1.3.1.2 Engineering bioremediation

Engineering bioremediation involves a process where bioremediation performance is enhanced artificially. Aerobic *in situ* bioremediation of contaminant is sometimes limited by the dissolved oxygen tension. Methods such as air sparging, injection of oxygen-releasing compounds, e.g. hydrogen peroxide, and trapped gas phases have been used to increase

dissolved oxygen concentration (Landmeyer and Bradly, 2003; Yang *et al.*, 2005). Some results of monoaromatic removal from contaminated site through enhanced in situ bioremediation are summarised in Table 1.2.

Table 1.2: Published studies of monoaromatic removal from contaminated site through enhanced in situ bioremediation

Contaminant	Electron acceptor (s)	Result (s)	Reference
Benzene (petroleum)	Sulphate and Fe(III) anaerobic	Results reviewed that addition of sulphate can be an effective strategy for enhancing anaerobic benzene removal. Addition of sulphate (in a short term incubation: less 2weeks) slightly stimulated benzene degradation and caused a small decreased in the ratio of methane to CO_2production from benzene. While addition of sulphate (in a long term incubation: more than 100 days) significantly stimulated benzene degradation with complete shift of CO_2 at the end product of benzene degradation.	Weiner *et al.*, 1998
BTEX gasoline	sulphate anaerobic	Injection of sulphate increased the rate of biodegradation of BTEX.	Sublette *et al.*, 2006
BTEX petroleum	Nitrate anaerobic	Nitrate addition resulted in loss of BTEX after initial lag phase 9days. Benzene losses were not observed over the 60 days period.	Schreiber and Bahr, 2002
BTEX gasoline	Oxygen	Oxygen enhanced zone was able to biodegrade benzene and ethyl benzene which were relatively resistant to natural attenuation.	Gibson *et al.*, 1998
BTEX petroleum	Nitrate and sulphate anaerobic condition	BTEX removal was increased after injection of nitrate and sulphate as compared to natural attenuation. Degradation of total xylenes appears linked to sulphate utilization.	Cunningham *et al.,* 2001
Benzene (petroleum)	Fe (III) anaerobic	Benzene and toluene were biodegraded when chelated Fe (III) was used as the terminal electron acceptor.	Caldwell *et al.,* 1999

1.3.2. *Ex situ* bioremediation processes

Ex situ bioremediation is the process whereby organic pollutants are removed and treated elsewhere from the polluted site (Aggarwal *et al.,* 1990). *Ex situ* treatments include land farming, compositing, bioslurry and biopiling (Rhykerd *et al.*, 1999). Soil biological

activities: microbial counts, soil respiration, soil biomass and enzymes activities can be used to ascertain the extent of bioremediation process of oil contaminated soil (Margesin *et al*., 2000). Diplock *et al.,* (2009) suggested that meeting a bioremediation end point could be used to evaluate and predict the bioremediation of a contaminate. The biochemical potential for contaminates to reach a target level is used to define the end point of the remediation process (Bundy *et al.,* 2004). However, Joergensen *at al.,* (1995) disagreed that it is not sufficient enough to only make use of the remaining hydrocarbon content in the soil to access the results of biological decontamination because not all minerals are completely mineralized to carbon dioxide during biodegradation. Quantification of the total culturable degrader population; an evaluation of hydrocarbon bioavailability and a measure of constraints to biodegradation are key parameters to predict the likely performance of a bioremediation strategy Paton *et al.* (2005). Lin *et al.* (2010) used a bioprocess of bioaugmentation and biostimulation with a molecular monitoring microarray biochip integrated with land farming operation to effectively degrade approximately 70% and 63% of diesel oil and fuel oil respectively after a period of 28 days. The bioslurry assays yielded a total petroleum hydrocarbon (TPH) reduction efficiency of 57% and 65% in 28 days respectively Frutosa *et al.* (2012). Radwan *et al.* (2002) employed the technique of immobilizing oil degrading bacteria nocardioforms and *Acinetobacter* in biofilms coating macroalgae to remediate n-octadecane and phenanthrene while Kermanshahi *et al.* (2005) used immobilized microbial cell airlift bioreactor for aerobic bioremediation of simulated diesel fuel contaminated groundwater .Gargouri *et al*. (2011) used a continuously stirred tank bioreactor (CSTR) to optimize feasible and reliable bioprocess system to treat hydrocarbon-rich industrial wastewaters. The use of the mixed cultures in the studies demonstrated high degradation performance for hydrocarbons range n-alkanes (C_{10}–C_{35}). At 225 days, it was observed that the residual TPH decreased from 320 mgTPH l^{-1} to 8 mgTPH l^{-1}.

1.4 Distribution of petroleum hydrocarbon utilising microorganism

Hydrocarbon degrading microorganisms are widely distributed in freshwater, marine and the soil environment, as summarised in Table 1.3. Cyanobacteria have also been associated with the degrading hydrocarbon (Chailana *et al.,* 2004; Lliros *et al.,* 2003). *Pseudomonas spp* has been involved in bioremediation of phenolic compounds; theses include *Pseudomonas aeruginosa, Pseudomonas fluorescence.* Microbial communities in contaminated soils are dominated by strains of bacterial that can survive toxicity and use the contaminant for growth (Zucchi *et al.,* 2003). A study has revealed the high potency of *Pseudomonas* strains in bioremediation of petrochemical waste-waters (Ojumu *et al.,* 2004). Typical groups of bacterial known for their ability to degrade hydrocarbon include: *Micrococcus, Pseudomonas, Alcanivorax, Microbulbifer, Cellulomonas*. Hexadecane was degraded by *Pseudomonas putida, Rhodococcus erthroplolis* and *Bacillus thermoleoovorans* (Ahmed Abdul-Megeed *et al*., 2010).

Table 1.3: Published studies of hydrocarbon utilizing microorganisms

Microorganisms	**Hydrocarbon**	**Reference**
Methylococcus, Methylomonas Methylosinus and Methylocella	C_1–C_8 alkanes alkenes and cycloalkanes	McDonald *et al.* (2006)
Pseudomonas, Burkholderia Rhodococcus and Mycobacterium	C_5–C_{16} alkanes, fatty acids, alkyl benzenes and cycloalkanes	Jan *et al.* (2003)
Candida maltose, Candida tropicalis and Yarrowia lipolytica	C_{10}–C_{16} alkanes, fatty acids	Iida *et al.* (2000)
Acinetobacter, Caulobacter and Mycobacterium	C_5–C_{16} alkanes, cycloalkane	Van Beilen *et al*.(2006)
Acinetobacter	C_{10}–C_{30} alkanes	Maeng *et al.* (1996)
Rhodococcus sp.	Pyrene, fluoranthene	Grosser, *et al*. (1991)
Xanthamonas sp.	Pyrene, benzo(a)pyrene	Grosser, *et al*.(1991)
Arthrobacter sp.	Fluorene	Casellas *et al.* (1997)
Bacillus cereus	Pyrene	Kazunga *et al.* (2000)
Rhodococcus erythropolis	Alkylated dibenzothiophene	Folsom *et al*. (1999)
Rhodococcus sp.	Napthalene	Walter *et al*. (1991)

1.5. Mechanism employed for hydrocarbons degradation

The most complete and effective degradation of the majority of the hydrocarbon and related contaminants is brought about under aerobic conditions. Figure 1.1 shows the main principle of aerobic degradation of hydrocarbon. The activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidises. The starting intercellular attack of organic contaminants is an oxidative process. Peripheral degradation pathways convert organic contaminants step by step into intermediates of the central intermediary metabolism, for instance the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for instance acetyl-CoA, succinate and pyruvate. Sugars required for various biosyntheses and growths are synthesized by gluconeogenesis (Fritsche and Hofrichter, 2000).

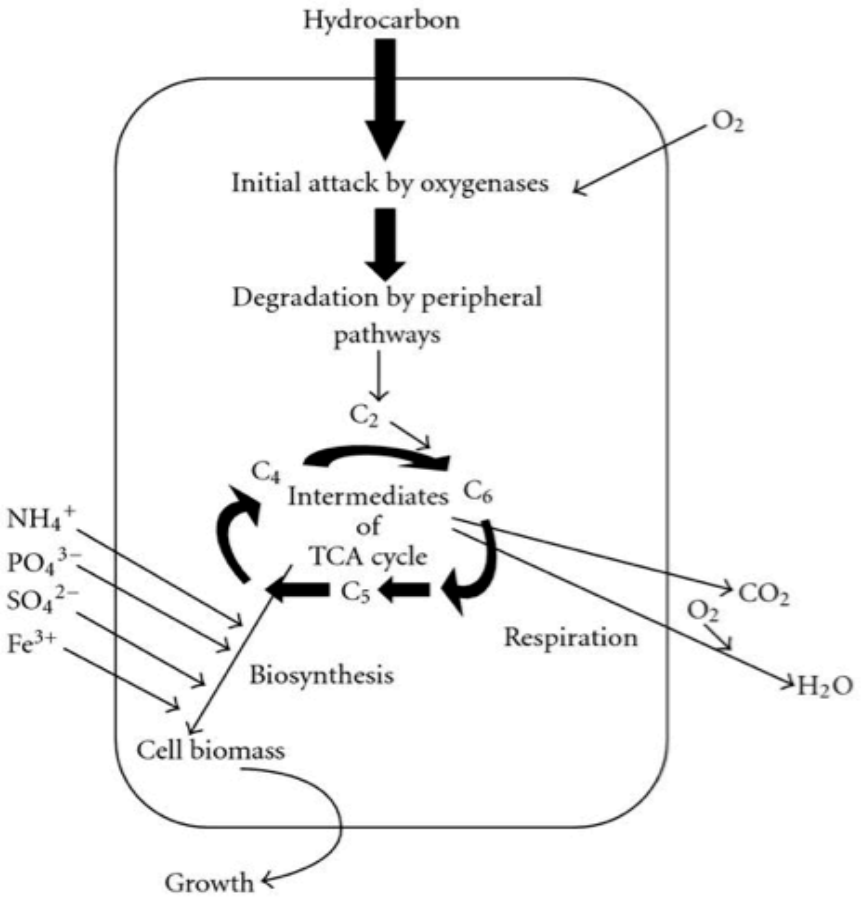

Figure 1.1: Main principle of aerobic degradation of hydrocarbons by microorganism
(Source: Das and Chandran (2010), *Biotechnology Research International*)

1.5.1. Uptake of petroleum hydrocarbons by biosurfactant

Mulligan, (2005) described biosurfactants as surfactants that are produced extracellularly or as part of the cell membrane by bacteria, yeasts and fungi. Biosurfactants has a toxicity, biodegradability and effectiveness in enhancing biodegradation and solubilization of low solubility compounds. Nikolopoulou and Kalogerakis (2009) further explained that biosurfactants increase the oil surface area and that amount of oil is actually available for bacteria to utilize it. Some other biosurfactants of interest are shown in the Table 1.4. Some particular type of biosufactant (e.g. glycolipid) can be produced by different bacteria (e.g. *Aeromonas spp and Bacillus spp*) and some bacteria genus can produce more than one biosufactant (e.g. *Acinetobacter spp*, *Bacillus spp*).

Table 1.4: Published studies of different biosurfactants produced by hydrocarbon degraders

Microorganisms	**Biosurfactants**	**Reference**
Pseudomonas aeruginosa	Rhamnolipids	Kumar *et al.*, 2008
Candida bombicola	Sophorolipids	Daverey and Pakshirajan, 2009
Pseudomonas fluorescens	Rhamnolipids	Mahmound *et al.*, 2008
Bacillus subtilis	Surfactin	Youssef *et al.*, 2007; Mulligan, 2004
Aeromonas spp	Glycolipid	Ilori *et al.*, 2005
Bacillus spp	Glycolipid	Tabatabaee *et al.*, 2005
Acinetobacter radioresistens	Alasan	Toren *et al.*, 2001; Navon-Venezia *et al.*, 1998
Acinetobacter calcoaceticus	Alasan	Taylor and Juni, 1961
Acinetobacter spp	Emulsans	Taylor and Juni, 1961

Mulligan *et al.* (2004) carried out a feasibility study of using biodegradable surfactant-surfactin from *Bacillus subtilis* to remove heavy metals from contaminated soil and sediment. Considerable research has been conducted on rhamnolipid biosurfactant produces by various

pseudomonas aeruginosa strains able to selectively complexing cationic metal species such as Pb, Cd and Zn (Herman *et al.*, 1995). *P.aeruginosa* ATCC9027 produced 5mM solution of rhamnolipid, which was found to complex 92% of cadmium, a complexation of 22 μg/mg rhamnolipid (Tan *et al.*, 1994). They were used to improve the bioavailability of crude oil, diesel, or PAHs (Bordoloi and Konwar, 2008; Providenti *et al*., 1995). Chen *et al*. (2013) observed that rhamnolipids enhanced oil degradation efficiency by 5.6% and had a different impact on the degradation of different petroleum hydrocarbon.

The biosurfactant, rhamnolipid produced by *Pseudomonas aeruginosa* utilizes hydrocarbon and organic contaminant and the process involved in the hydrocarbon utilization is illustrated in Figure 1.2.

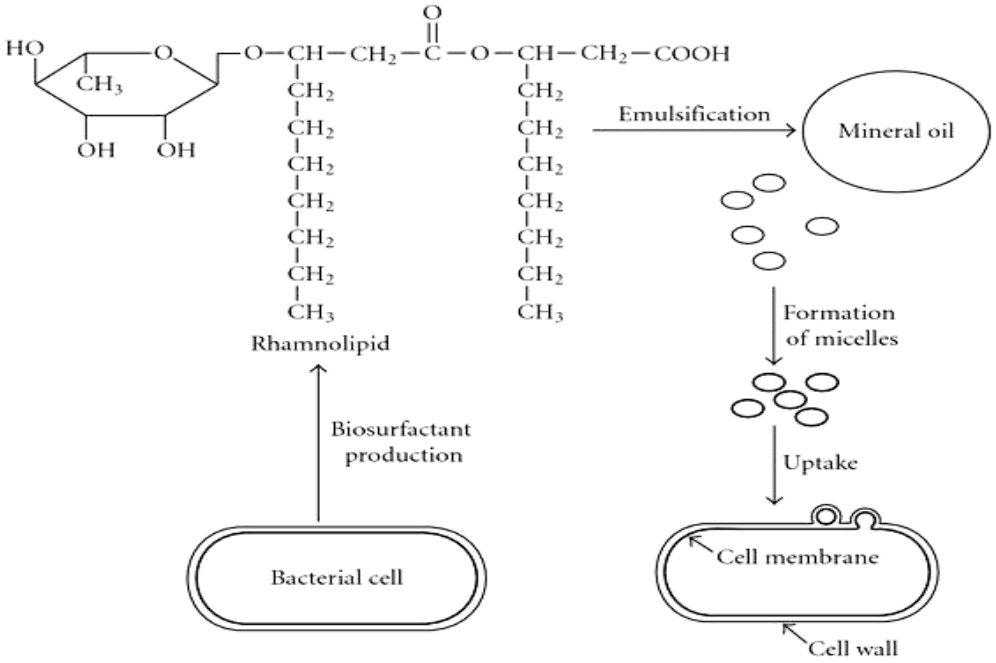

Figure 1.2: show how rhamnolipid a biosurfactant produced by *Pseudomonas sp* utilizes hydrocarbons
(Source: Das and Chandran (2010), *Biotechnology Research International*)

Other mechanisms involved are attachment of microbial cells to the substrates and production of biosurfactants. The uptake mechanism linked to the attachment of cell to oil droplet is stillunknown but production of biosurfactants has been researched on (Das and Chandran, 2010).

1.6 The Niger Delta region and oil spills.

The Niger Delta region is situated around the tributaries of the Niger River into the Southern Atlantic Ocean and the core south-south of Nigeria **(see figure 1.3)**. The region is made up of wetlands, creeks and small islands. Its ecological zone is made up of a coastal barrier sandy ridge, mangrove swamp, freshwater swamp, and lowland rainforest. The people's predominant occupation in the region is agriculture and fishing (Francis *et al.,* 2011).

The Niger Delta region is where oil production takes place is considered as one of the major oil-exploring region of the world (Sojinu *et al.,* 2010). Since discovery of the first oil well in the region at Oloibiri in 1956 by Shell-British Petroleum, oil exploration has led to the releases of various pollutants including trace elements and poly aromatic hydrocarbon into the environment **(see figure 1.3)**.

Figure 1.3 shows an oil spill on farmland in Ikot Ada Udo village, Niger Delta, Nigeria.
(Source: Okop, I.J and Ekpo, S.C**,** (2012) *World Congress on Engineering*)

1.6.1 Soil types in the Niger Delta region of Nigeria

This region is underlined by different superficial soil deposit ranging from the organic mostly peaty marine mud or chicoco in the tidal flat or saline mangrove swamp to the active or montmorillonitic silty clay of the freshwater back swamps deltaic lateritic soils of the dry flatland or plains (Leton and Omotosho, 2004). The soil texture in region varied widely from sandy to predominantly silty loam, and sandy clay loam with a mixed clayey/silty loam texture (Effiong and Ayolagha ,2010) and are relatively acidic (Ayotamuno *et al.,* 2006).

Akpokodje (1987) explained that the superficial soil of the Niger Delta is classified into four major groups as shown in Table 1.5: a) Reddish brown sandy clay loam soil of low medium plasticity – (RBSCL-1), b) Brown sandy clay of medium plasticity – (BSC-2), c) Light grey, slightly organic, fine sand and silty clay – (LGFSC-3) and d) dark, organic/peaty clay of high extremely high plasticity – (DOPC-4).

Table 1.5 Textural classifications of the major soil groups in the Niger Delta region

SOIL TYPES	SOIL TEXTURE AND PARTICLE SIZES, %					
	Gravels, > 2 mm	**Coarse to medium sand, 125 µm – 2 mm**	**Fine sand, 63 -125 µm**	**Silt, 2 - 63 µm**	**Clay, < 2µm**	**Soil classification**
RBSCL - 1	0 - 4	42 - 77	17 - 30	1 - 3	15 – 26	Sandy loam, sandy clay loam
BSC - 2	0	21 - 51	15 - 17	5 - 16	26 – 56	Sandy clay loam, sandy clay
LGFSC - 3	0	0 - 8	7 - 23	17 - 22	44 - 61	Clay
DOPC - 4	0	0 - 2	0 - 2	2 - 21	47 - 66	Clay

(Source: Akpokodje, E.G. (1987) *Journal of Engineering Geology*, pp. 201)

Olorunfemi (1987) investigated the mineralogical composition of the Niger Delta surface sediments from localities as represented in Table 1.6. This was a statistical representative of soil from the region.

Table 1.6: The mineralogical composition of the soil samples from the Niger Delta region of Nigeria

SAMPLING AREAS WITHIN THE REGION	PERCENTAGE OF MINERALS IN THE REGION						
	Kaolinite	**Quartz**	**Smectite**	**Illite**	**Gibbsite**	**Allophane**	**Mica**
Abasmol	18	64	5	-	8	5	-
Umakroske soil port Harcourt	20	75	5	-	-	-	-
Owerri sand quality	5	95	-	-	-	-	-
Calabar	40	55	-	-	-	-	5
Eggamin Ndele	10	82	8	--	-	-	-
Oboburuchy	25	55	5	15	-	-	5

(Source: Olorunfemi, B.N. (1984) *Journal of African Earth Sciences*)

The findings from the studies review that the major minerals found in the region are quartz and kaolinite with some proportions of smeetite, goethite and gibbsite. The central delta is essentially quartz – kaolinite zone while the eastern delta has the highest concentration of highly aluminous smectite. Kaolinite has been shown to increase the rate of oil breakdown by bacterial digestion based on the report of Chaerum and Tazaki (2005). Studies have shown that clay and clay minerals are used in environmental protection and remediation (Murray, 2000; Churchman *et al*., 2006). Colloidal sized clays are used to aid dispersion in hydrocarbon-polluted environment (Meyer and Quinn, 1973; Owen and Lee, 2003). This finding is key in formulating the soil composition for this research investigation.

1.7 Zeolite properties

Gottardi and Galli, (1985) described zeolite as tectosilicates with three-dimensional alumino-silicates structure containing water molecules, alkali and alkaline earth metals in their structural framework. High specific areas and high cation exchange are important properties used to characterize zeolite (Breck, 1974). Virkutyte *et al*. (2002) describes zeolite as crystalline aluminium-silicates, with group I or II elements as counter ions. Structurally,

zeolite has a framework consisting of $(SiO_4)^{4-}$ and $(AlO_4)^{5-}$ tetrahedral linked to each other at a corner sharing oxygen. Zeolite has very high ion exchange, adsorbing, catalytic, capacities for molecular sieving. It is negatively charged (anionic) as such can be used to remove cations from aqueous solution. Amongst the different zeolites, clinoptilolite is the most abundant natural zeolite in the world (Wang and Peng, 2010). Natural zeolites in the past decades have been reported to have several applications such as adsorption, building industry, catalysis, energy, and agriculture and soil remediation. Clinoptilolite have been used to remove cationic radioactive species (^{137}Cs, ^{90}Sr) from nuclear plant wastewaters and contaminated groundwater's (Gunter and Zanetti, 2000; Cantrell *et al*., 1994).

1.7.1 Zeolite and soil remediation

In recent years, natural zeolite and its modified forms have been reported to used for the removal of anions and organic from waste water systems (Wang and Peng, 2010). Little to no information has been reported with regards the combined influence of bacteria and zeolite in remediating the soil environment of contaminated crude oil, which this research work will be investigating. Soil remediation is based on two approaches: extraction/removal of heavy metals from the matrix by electrokinetic and/or washing processes or reduction of metal mobility with *in situ* techniques such as phytoremediation (Virkutyte *et al.,* 2002). Researchers have argued that remediation of soils was majorly affected by the cation exchange capacity rather than the pH value. However some scientists consider pH value as the main factor through the smart leaching design (Wang and Peng, 2010).

1.7.2: Adsorption and diffusion properties of hydrocarbons in zeolite

Zeolite has been used as a catalyst and an adsorbent in many chemical and petroleum processes (Breck, 1975). An understanding of molecules adsorption and diffusion within the pores of zeolites is key in utilizing them for the design of new application materials (Ruthven, 1984; Chen *et al*., 1994). Studies have suggested that zeolite can be used in trapping

hydrocarbon (Yang and Kung, 1994). However, there are some suggestions that whilst the heavy exhaust hydrocarbons, e.g. aromatics, are adequately trapped by zeolites, the light hydrocarbon components of the exhaust often desorbs from the hydrocarbon trap before the catalytic process at high temperature (Czapleweski *et al.,* 2002).

Song *et al*. (2007) investigated the adsorption and diffusion behaviour of hydrocarbons: C_1-C_6 alkanes, C_5-C_8 cyclic hydrocarbon – benzene, toluene, p-xylene, ethylbenzene; in siilicalite-1, theta-1 and $AlPO_4$-5 using gravimetric balances, computer simulation calculations and the frequency-response (FR) technique respectively. They established that the adsorption of cyclic hydrocarbons in silicalite-1 reviewed anomalous adsorption properties while all the C_1-C_6 *n*-alkane molecules present simple type-1 isotherms in both silicalite-1 and theta-1 zeolite. Lemic *et al*., 2007 also carried out a competitive adsorption study of polycyclic aromatic hydrocarbons organo-zeolites to determine sorbent-sorbate interaction. They observed that at a concentration of 20 $\mu g/dm^3$, benz(a)anthracene had an adsorption index of 100%.

Extensive studies have been conducted using adsorbents such as granular activated carbon to bind PAHs and remove contaminants from water. Clays and zeolites have also shown effective adsorbents for a number of organic contaminants. Zeolites that occur as millimetre or greater sized particles exhibit superior hydraulic characteristics and are free of shrink-swell quality that is associated with clay (Song *et al*., 2007).

Carmody *et al*. (2007) used organo-clays synthesised by ion exchange in three surfactants: $C_{21}H_{46}BrN$, $C_{22}H_{48}BrN$ and methyl ammonium chloride to test for hydrocarbon (diesel, hydraulic oil and engine oil) adsorption. They concluded that hydrocarbon sorption capacity of the organo-clays depended upon the material and surfactants used in the organo-clay synthesis. They further explained that greater adsorption with organo-clays is obtained if the surfactant contained two or more hydrocarbon chains. Sharmasarker *at al*. (2000) earlier

demonstrated how organo-clays could be used to contain environmental pollutants from waste site and accidental spills. The study showed that organo-clays with smaller cations had greater hydrocarbon retention. Wefer-Roehl and Czurda (1997) established that organo-clays were useful for the adsorption of both aliphatic and aromatic hydrocarbon. Benzene, toluene and ethyl benzene were absorbed using hectorites (Jaynes and Vance, 1996).

1.8 Factors affecting bioremediation processes

There are numbers of limiting factors that have been known to affect the biodegradation of petroleum hydrocarbon. Theses include:

1.8.1 Effect of temperature on bioremediation

Temperature controls the bioavailability of low-solubility hydrocarbon, nature and the extent of microbial metabolism (Margesin and Schinner, 2001). Small increase of temperature (2 ºC) has a significant impact on alkane degradation (Delille *et al.*, 2004). In soil environment, the highest degradation rates generally occur in the range of 30-40 ºC while in some freshwater environments is 20-30 ºC and in marine environment is 15-20 ºC (Okoh, 2006). Bacteria in the soil have an optimum temperature ranging from 25 – 45 ºC (Nester *et al.,* 2001). High temperature enhances hydrocarbon degradation rates and biostimulants effect (Perfumo *et al*., 2007). Increased temperature results in a decrease of viscosity, higher solubility and faster diffusion of hydrophobic contaminants and enhances the rate biodegradation. (Sorkhoh *et al.,* 1992).

Si-Zhong *et al.* (2009) reported that ambient temperature influences the physico-chemical composition of oil, rates of hydrocarbon degradation and the composition of the microbial communities. Wang *et al.* (2001) added that while optimizing the conditions for hydrocarbon metabolism showed how temperature is vital and influence the rate of biodegradation. The

optimum temperature for biodegradation is usually 15-30 ºC for aerobic processes and 25-35 ºC for anaerobic processes (Si-Zhong *et al.,* 2009).

Antizar-Ladislao *et al.* (2004) reported that temperature had a significant influence in the removal of PAH, with a removal percentage rate of 60.8% at 38 ºC after a period of six weeks. Antizar-Ladislao *et al.* (2006) further investigated the influence of different programmes on the bioremediation of PAH and found out that using thermophilic (70 ºC) bacteria at the end of in-vessel compositing processes resulted in greater PAH removal than using other variable temperature profiles when the temperature increase is applied stepwise.

1.8.2 Microbial effects to bioremediation processes

The potential to degrade and utilize oil hydrocarbons has been observed in a number of bacteria, fungi and yeast, e.g. *Candida, Saccharomyces*, some cyanbacteria e.g. *Oscillatoria, Anabaena Phormidium, Scenedesmus* and green algae, e.g. *Chlorela, Microcoleus, Ulva*. Bacteria are mostly used during soil bioremediation because they are distinguished by high frequency, fast growth and a wide spectrum of the utilized petroleum products (Wolicka *et al*., 2009). *Methylomicrobium sp* has the potential of oxidizing halogenated hydrocarbon (Margesin and Schinner, 1999).

Sorkhoh *et al.* (1992) investigated 38 samples obtained from the oil-contaminated sites in Kuwait and found out that all the 368 isolates were from the genus *Bacillus* and the predominant species were *Bacillus stearothermophilic*. Two strains degraded about 80-89% of the crude oil (5 gl^{-1}) within 5 days at their optimum growth temperature of 60 ºC. The microbial activity of *Photobacterium phosphoreum T3* increased remarkably while hydrocarbon degradation in contaminated soil was enhanced as a result of stimulation of microbial population size and activity from high rate of organic amendment (Liu *et al.* 2009).

Philips and Atlas (2005) reported that a number of researchers have postulated that the ability of inoculums to degrade recalcitrant compound is relatively depended on the size of the inoculums. For contaminated soils the numbers of inoculums have been highly variable. Ijah and Antai (2003) earlier observed that the counts of hydrocarbon degraders in oil polluted soil to be 10^3 cfu/g. Microbial communities in a contaminated soils is dominated by strains of bacteria that can survive toxicity and are able to make use of the contaminants for growth. As a response to bioremediation treatment, these populations may begin to actively degrade the pollutants and detoxify the soil, allowing other starving populations to increase their numbers, leading to an increase of bacterial community in the soils (Zucchi *et al.,* 2003).

1.8.3 Soils influence on microbial degradation of hydrocarbon

The soil environment is the most dynamic site of interactions in nature (Scherr *et al.,* 2007). Microbial community carries out the majority of decomposition processes in the soil and are irreplaceable in the transformation and degradation of synthetic organic compound and natural waste material Obire *et al*. (2001). Scherr *et al.* (2007) observed that freshly spiked silty soil showed higher degradation of about 51% as compared to sandy soil with 25% degradation when recently contaminated with hydrocarbon. It was further explained that degradation occurred in fine soil fraction with a high content of organic matter. Soil is a mixture of mineral and organic materials and is an effective adsorbent for PAHs (Harayama, 1997). Soil permeability and pore and fractures in the rock are critical factors controlling the movement of water from the surface into the underlying layers (Atlas *et al.,* 2005)

The permeability of soil to air and water is a function of soil texture. Fine-textured soils like clays have low permeability, which prevents biovented oxygen and nutrients from dispersing throughout the soil. Fine textured soils are slow to drain from water-saturated soil condition, thus preventing oxygen from reaching soil microbes throughout the contaminated area (USEPA, 2006).

Clay minerals increase the rate of bacteria growth and influence hydrocarbon degradation (Van Loosdrecht *et al.,* 1990; Chaerun and Tazaki, 2005). Stotzky and Rem (1966) suggested that smecties buffering ability and their potential to adsorb proton released during the breakdown of hydrocarbon helps to maintain optimal pH conditions and sustain bacterial growth. Kaolinite has been shown to influence oil breakdown by bacterial digestion (Chaerun and Tazaki, 2005). Soils with sandy texture and low organic carbon are known for poor microbial proliferation and diversity brings about lower degradation rates as compared to clay loam and loam (Talley *et al.,* 2002; Hejazi *et al.,* 2004). The low amount of degraders in the sand fraction of contaminated soils is mutually related to a higher carbon/nitrogen ratio and lower internal surface (Amellal *et al.,* 2001). In as much as the soil microbial community benefits from high levels of soil organic matter in its size, diversity and ability to recover from environmental and contamination stresses; the soil organic matter is found to be inversely proportional to degradation rates (Trindade *et al.*, 2005).

1.8.4 Influence of oxygen availability and transport on bioremediation

Oxygen is a key component to bioremediation processes and its delivery is crucial to the success or failure of bioremediation processes. Water saturation of soil pores slows oxygen transport to very low levels. Soil contaminated with 10 m^3 of hydrocarbon would require about 2 x 10^6 m^3 of water saturated at 10 mg/litre O_2 for effective biodegradation (Atlas and Philip, 2005). In soils the oxygen content depends on the microbial activity, soil texture, water content and soil depth. Mineralization of hydrocarbon from soil was severally limited when the oxygen content was below 10% (Vasudevan and Rajaram, 2001).

Devinny and Islander (1998) observed that oxygen diffusion limits the performance of land treatment units only in cases of high respiratory activity. The importance of oxygen comes from the participation of oxygenases and molecular oxygen involved in the major degradation pathways for the hydrocarbons. Theory suggests that the mass of oxygen necessary to

remediate the hydrocarbon load is about 0.3 g oxygen for each gram of oil oxidized (Atlas and Bartha, 1973). The supply of oxygen is a common constraint to the bioremediation in frozen ground because oxygen is scare and the diffusion of oxygen is partly or fully blocked. (Si-Zhong *et al.,* 2009).

Lin *et al.* (2012) used an oxygen–releasing bead (ORB) and oxygen –releasing immobilized cell bead (ORICB) investigate oxygen effect on degradation of BTEX contaminated groundwater in a column and reported that the encapsulated-ORBs had much higher oxygen-releasing capacity and a longer effective oxygen releasing period than the mixed-ORBs and the BTEX removal rates increased as flow distance and the increased in the influent BTEX concentrations altered the indigenous microbial communities.

1.8.5 Hydrocarbon concentration impact on bioremediation processes

Petroleum biodegradation is highly dependent on environment conditions and on the chemical structure of the pollutant compounds (Swannell *et al*., 1996; Aldrett *et al.,* 1997). The rates of degradation and the quality of oil eliminated also depend on the type and amount of oil present at the contaminant site. (Del Arco and de Franca, 2000). A number of authors have carried out studies on contaminated soils with initial oil concentrations between 0.7 g/kg and 75.0 g/kg (Wang and Bartha, 1990; Geerdinket *et al.*, 1996;). De Jonge *et al*. (1997) cited 4 g/kg as a boundary value between low and high levels of the referred parameter. Del Panno *et al.* (2005) reported that there were qualitative and quantitative changes of microbial communities in soil microcosm during bioremediation when soil were contaminated with 1.0%, 2.5%, 5% and 10% (wt/wt) of petroleum sludge containing PAHs and monitored for a year period. The changes in the community structure of soil depended on the amount of added sludge and the predominant species in the sludge community could not be detected at the end of the investigation.

Soil contamination as a result of PAHs is sometimes associated with the presence of high levels of potentially toxic metals (Allieri *et al.*, 2005). A wide range of metal concentrations e.g. zinc, cadmium, chromium (III and VI), nickel, mercury and lead has inhibited organic biodegradation. The least concentration of cadmium (Cd) reported to reduce biodegradation of PAHs ranged from between 0.002 and 1 mg/L (Springael *et al*., 1993; Sandrin *et al*., 2000) in terms of free Cd species. Maslin and Maier (2000) observed a 5-day increase in lag period for phenanthrene degradation in the presence of 1 and 2 mg available Cd/L and complete inhibition at 3 mg available Cd/L. Sayara *et al.* (2010) reported that the degradation rates of a contaminant increased with the increase in the PAHs concentration. However, Jorgensen *at al*. (2000) did not fully agreed with this as the microbial activity could be affected or inhibited when high concentration of contaminant is available as affecting the rate of degradation.

1.8.6 Effects of nutrient on biodegradation

Lieberg and Cutright (1999) used a respirometer to determine the effect of adding macro and or micronutrients for enhancing the bioremediation of PAHs in contaminated soils. They reported that the optimal oxygen consumption resulted when a low level of macronutrients and a high level of micronutrients were used with phosphorus as the dominant macronutrient and the bioactivity of the foreign consortium was the greatest when a high level of micronutrients was used. Kwok and Loh (2002) used the nutrients - ammonium nitrate, KH_2PO_4, phenyl phosphate to enhance biodegradation of phenanthrene by *Pseudomonas putida* in six synthetic Singapore soil and reported that that increasing KH_2PO_4 increased the biodegradation rate of phenanthrene across all soil types, indicating that phosphorus was limiting. Biostimulation by nutrient application is an effective technology for restoring oil-contaminated beaches. Nutrient concentrations sufficient for maximum growth of hydrocarbon degrading microorganisms are very small 2.0 – 10.0 mg/L (Li *et al*., 2007). The nutrient level of a soil directly impacts microbial activity and biodegradation. (Si-Zhong *et*

al., 2009). Inorganic nutrients are not just necessary for microbial activity but for cell growth. Nitrogen can increase cell growth rate, decrease the microbial lag phase, help to maintain microbial population at high activity levels and increase the rate of hydrocarbon degradation. Excessive amount of nitrogen in soil cause microbial inhibition as such maintaining nitrogen level below 1800 mg N/kg H_2O for optimal biodegradation of petroleum hydrocarbon (Walworth *et al.*, 2005).

1.9 Aims of this thesis

The research is aimed at developing an effective bioremediation strategy for petroleum hydrocarbon that is cheap, easily managed and applicable for warm climate. Detailed objectives are to:

1. Isolate and screened bacteria from culture collections and study hydrocarbon degradation rates in warm climates.
2. Evaluate the success of bioagumentation for high temperature and study the impact of variation in temperature and hydrocarbon concentration on the growth and survival of bacteria.
3. Assess the effects of different types of soil (clayey and sandy) on the biodegradation rates of hydrocarbons and to evaluate the effectiveness of addition of zeolites on bioremediation.
4. Recommend a bioremediation strategy for hydrocarbons that is cheap, easily managed and applicable for warm climates.

CHAPTER 2

GENERAL METHODS AND MATERIALS

2.1 Preparation of basal salt medium (BSM)

The BSM was prepared by first preparing separate stock solutions of the following chemicals: 50 g/250 ml K_2HPO_4, 50 g/ 250 ml KH_2PO_4, 50 g/ 250 ml KNO_3, 50 g/ 250 ml $(NH_4)_2SO_4$, 50 g/ 250 ml NaCl, 50 g/ 250 ml $MgSO_4.7H_2O$. These concentrations were prepared by using the recipe of Karamalidis *et al*., 2010, as follows and adopting K_2HPO_4 as an example; 50 grams of K_2HPO_4 was weighed separately using a weighing balance into a 250 ml beaker and 100 ml distilled water was added. The solution was stirred using a magnetic stirrer to ensure the compound was completely dissolved. The solution was then poured into a 250 ml volumetric flask with the aid of a funnel and made up to the 250 ml mark of the volumetric flasks with distilled water, thus producing a stock solution 50 g/ 250 ml. The same procedure was repeated in the preparation of the remaining chemicals.

Stock solution of the trace elements: 2 g/ 50 ml $CaCl_2$, 2 g/ 50 ml $CuSO_4.5H_2O$, 2 g/ 50 ml $MgSO_4.5H_2O$, 2 g/ 50 ml $ZnSO_4.5H_2O$, 2 g/ 50 ml $(NH_4)_6Mo_7O_{24}.4H_2O$ and 2 g/ 50 ml $FeSO_4$ were make adopting the recipe of Karamalidis *et al*., 2010. The composition of trace element solution was prepared by transferring 50 µm of the each of the trace elements stock solution by pipette into 1-litre volumetric flask and made up to the designated mark with distilled waters. Finally, 10 ml each of BSM stock solution chemical prepared were individually transferred into 1litre volumetric flask using a pipette except for $MgSO_4.7H_2O$ and NaCl which 1 ml each were transferred. Then 10 ml of the trace elements mixture was added into the 1 litre volumetric flask and made up to the designated mark with distilled water. The BSM final solution consisted of 1.0 g/l K_2HPO_4, 1.0 g/l KH_2PO_4, 1.0 g/l KNO_3, 1.0 g/l

$(NH_4)_2SO_4$, 0.1 g/l NaCl, 1.0 g/l $MgSO_4.7H_2O$, 2 mg/l $CaCl_2$, 2 mg/l $CuSO_4.5H_2O$, 2 mg/l $MgSO_4.5H_2O$, 2 mg/l $ZnSO_4.5H_2O$, 2 mg/l $(NH_4)_6MO_7O_{24}.4H_2O$ and 2 mg/l $FeSO_4$.

2.2 Preparation of bacterial cultures

The freezed dried ampoules of bacteria isolates from UWCC were cultured on trytone soy agar (TSA), 0.1% hexadecane agar (HA) and 0.1% sodium benzoate (SB) agar plates. These were incubated at 30 ^oC for 48 h (TSA plates) and for 96 h (for hexadecane and sodium benzoate agar plates). Isolated bacteria colonies were subsequently transferred into fresh plates of TSA, 0.1% HA and 0.1% SB cultured for 30 ^oC and at the period earlier described and stored in the refrigerator at 4 ^oC for further usage.

2.3 Identification of bacteria isolates

The following tests describe were used to verify the identity of the screened and selected bacteria for hydrocarbon degradation.

2.3.1 Gram stain

Heat fixed deposit of suspended bacterial cells in saline solution were flooded with crystal violet for 1 minute followed by Grams iodine for another 1 minute. The slide was drained with acetone and safarin was poured on the slide for 30 sec, after which the slide was rinsed with distilled water at each step. The slide was finally air-dried and examined under oil immersion objective.

2.3.2 Biochemical analysis

The following tests were used to verify the identity of the four selected bacteria isolates screened for hydrocarbon degradation.

1. Motility test

The wet mount procedure was used to determine the bacteria motility. A pipette was used to introduce 20 µl of the bacteria culture onto a clean scratch free glass slide. Then a clean cover

slip was carefully placed over the drop avoiding bubbles formation. The slide was examined first under 4 X magnification followed by 40 X and 100 X magnifications and motility determined by observation.

2. Oxidase test

One drop of oxidase reagent was pipetted onto a piece of filter paper. The cells of bacteria were then smeared across the surface of the impregnated filter paper with the aid of a glass rod and an instant development of a dark purple colour indicated a positive reaction.

3. Catalase test

One drop of 10% hydrogen peroxide (Sigma) was dropped on a slide then a loopful of bacteria introduced, an immediate effervescence indicated a positive result. The enzyme catalase protects bacteria from the toxic oxygen derivative hydrogen peroxide, which is produced by aerobic metabolism within the bacterial cell. The bacterial cells react directly with hydrogen peroxide and then observing the evolution of O_2 gas.

Table 2.1 Identification of selected bacteria isolates

	Selected hydrocarbon degraders			
	Pseudomonas aeruginosa	***Rhodococcus sp***	***Acinetobacter sp***	***Bacillus sp***
Motility	+	-	-	+
Gram reaction	-	+	-	-
Oxidase	+	+	-	+
Catalase	+	+	+	-
Shapes	Rods	Short rods	Rods like	Coccus

+ = Positive; - = negative

2.4 Bioscreen growth analyzer (BGA)

Growths of bacterial cultures were determined using the Thermo Labsystems Bioscreen C as described:

1 Preparation of standard inoculums

Individual bacterial was grown on BSM with a hydrocarbon (e.g. hexadecane) for 18 h at 30 °C in an orbit shaker at 150 rpm. Bacteria cells was harvested by centrifugation, rinsed three times in sterile saline before being re-suspended in 5 ml sterile liquid basal medium (BM) to yield an absorbance reading of 0.5 at 540 nm and the cell count was determined using the methodology of Ghazali *et al.*, (2007)

2. *Preparations of BSM + hydrocarbon solution*

Specific volumes each of BSM **(see Appendix I Table 2)** were measured into separate medical bottles, properly labeled and autoclaved at 121 ^{o}C for 20 minutes. After the autoclaving, specific volumes of hydrocarbon was measured and sonicated into the BSM Solution, producing a final solution (BSM + hydrocarbon) volume each of 9.98 ml. The equivalent volume of bacteria inoculum required to obtained concentration of hydrocarbon in the solution of BSM and hydrocarbon was prepared **(see Appendix I- Table I.2).**

3. Filling of bioscreen inoculum well.

280 µl of the BSM + hydrocarbon solution prepared was first introduced into the well of the bioscreen plate **(see Appendix I- Table I.3)** using the micropipette then the bioscreen was set up and adjusted to a wavelength of 540 nm. Readings were taken after every 1 h at a temperature of 30 ºC. Then 20 µl of the inoculum was aseptically pipetted into the wells in a Microflow biological safety cabinet before transferring the well into the bioscreen. The experiment ran for 5 days.

3. Bacterial growth rate determination.

The growth rate and mean generation time of the bacteria isolates on the hydrocarbon supplemented in BSM were determined from the data generated and calculated as shown in **(see Appendix I- Calculation I.1 and Calculation I.2)** for a sample.

CHAPTER 3

SCREENING OF BACTERIA FOR GROWTH ON PETROLEUM HYDROCARBON AND EFFECT OF HYDROCARBON CONCENTRATIONS ON BACTERIA GROWTH

3.1 INTRODUCTION

A total of twenty-one (21) bacterial isolates from the University of Wolverhampton Culture Collection (UWCC) were screened for hydrocarbon degradation using the plate method **(see Appendix I- Table I.1)**. Some of these bacteria were isolated from petroleum hydrocarbon sites and some were known hydrocarbon degraders. Temperature was the main parameter used to screen these bacteria. The choice of temperature (range 25-50 ^{o}C) was to establish the optimum temperature for the bacterial growth and survival on hydrocarbons. Hexadecane and sodium benzoate were the two hydrocarbons used for screening the bacterial isolates before further screening for petroleum crude oil. The choice of these hydrocarbons was because of their simple nature and to provide examples of straight alkanes and aromatics as carbon sources for growth, which will enhance the next phase of the research investigation.

The bacterial isolates (*Pseudomonas aeruginosa, Bacillus sp, Acinetobacter sp* and *Rhodococcus sp*) were selected from the twenty-one (21) screened isolates because of their optimum growth and survival at high temperature range. The selected bacteria were then evaluated for growth and survival at varying hexadecane concentrations (0.5-2.0%) and sodium benzoate (0.5%) using the bioscreen growth analyzer method. The shake flask method was subsequently used for the bacterial growth on the hydrocarbons and on petroleum crude oil because in adequate aeration and agitation experienced with the bioscreen growth analyzer. Three (03) bacterial isolates (*Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp*) were finally selected based on their individual level of growth on basal salt

media (BSM) supplemented with 1.0% petroleum crude oil and as a mixed culture for subsequent bioremediation studies. The aim of this aspect of research was to screen appropriate bacteria that could be used for subsequent bioremediation studies involving bioaugumentation of oil-contaminated soil in a warm environment.

3.2 METHODS

3.2.1. Collection of stock cultures

Bacterial stock cultures were obtained from the UWCC **(Appendix I- Table I.1)** and prepared as described **(Section 2.2).** However, the bacterial identities were then reconfirmed using methods described in **(Section 2.3).**

3.2.2 Preparation of basal salt medium agar (BSMA) with hydrocarbon

The BSM used was prepared as described in **(Section 2.1).** The hydrocarbon used for the investigation was supplied by: Fisher chemical (hexadecane), Sigma (Sodium benzoate) and Petroleum crude oil obtained from a Swansea Petroleum Depot, UK.

3.2.2.1 Preparation of BSMA

4.5 grams of Agar No 2 was weighted into a 500 ml Medical bottle and was homogenized with 300 ml BSM solution. The solution was then autoclaved at 121^{o}C for 20 minutes. The molten BSMA was kept in the incubator at 50 ^{o}C until required.

3.2.2.2 Preparation of basal salt medium agar + hexadecane

Homogenized mixture BSMA (20 ml) was poured into a 50 ml Erlenmeyer flask covered and wrapped around with an aluminum foil paper while the remaining mixture (480 ml) was autoclaved in the medical bottle. All were autoclaved at 121 ^{o}C for 20 minutes. 0.5 ml of hexadecane was aseptically poured into the beaker containing 20 ml sterile molten BSM agar. The beaker was then held in place in the chamber of a sonicator (Bandelin Sonoplus, Model 2200) via a restraining cable and snap lever on the clamp stand. The flat-tip (VS70T) of the sonicator probe was thoroughly cleaned using ethanol before being inserted to a depth of

about 10 mm of the mixture. The sonicator was then continuously operated for 5 minutes to create an emulsion. The sonicated mixture of sterile BSM agar with hexadecane were then aseptically poured into the autoclaved BSM agar in the medical bottle and thoroughly mixed. The molten mixture was then aseptically poured into sterile Petri dishes and left to harden.

3.2.2.3 Preparing basal salt medium agar + sodium benzoate

Sodium benzoate 5% (w/v) was prepared in 50 ml sterile BSM and sterilized by membrane filtration (0.45 μm). The filtrate was of the sterile sodium benzoate was added to 450 ml of BSMA at 50 ^{o}C, mixed by gentle inversion and plates immediately poured.

3.2.3 Screening bacterial isolates for hydrocarbon degradability.

Bacteria were screened for petroleum hydrocarbon degradation at temperatures range of 25-50 ^{o}C of hydrocarbon concentrations of 0.1%, 0.5%, 1.0% and 2.0%. These were carried out via culturing of the bacterial isolates on hydrocarbon containing BSMA and TSA plates. The plates were incubated in the incubators at average temperature of 24 ± 1^{o}C, 30 ±1^{o}C, 37 ± 1^{o}C and 50 ± 1^{o}C for a period up to 7 days.

3.2.3.1 Determination of bacterial growth on hydrocarbon media

The batch culture in shake flakes was used for the bacterial growth experiments. Samples were taken aseptically at 0, 48, 96, 144 and 192 h and the Miles and Misra technique was used to determine the colony forming units /ml (CFU/ml) in triplicates. Serial tenfold dilution from 10^{-1} to 10^{-8} was performed by dispensing 0.5 ml of sample in 4.5 ml of sterile 1/4th strength ringer solution. 20 μl of each dilution was dispensed onto TSA and hydrocarbon (hexadecane, sodium benzoate and crude oil) plates under aseptic conditions. The TSA plates were then incubated at 30°C for 24hrs whilst other plates were incubated for up to 144 h.

3.2.4 Determination of bacteria growth dynamics at different concentrations of hydrocarbon using the bioscreen growth analyzer (BGA)

The growth dynamics of the screened bacterial isolates were determined using a bioscreen growth analyzer as described in **(Chapter 2**: **Section 2.4.2).** Hexadecane concentrations of 0.5%, 1.0%, 1.5% and 2.0 % (w/v) and Sodium benzoate 0.5% (w/v) in BSM media were utilized in culturing screened bacteria.

3.2.5 Determination of bacterial growth in the presence of crude petroleum hydrocarbons: shake flask method (SFM)

The method of Das and Mukherjee (2007) was employed to determine the bacterial growth. Replicate batch bacterial culture were grown in 250 ml Erlenmeyer flasks containing sterile 100 ml mineral salts supplemented with crude petroleum hydrocarbon at a final concentration of 1.0% (v/v). Inoculation was performed at 30°C ± 1°C and at a pH of 7 with 250 rpm rotary shaking. Un-inoculated flasks and flasks without petroleum hydrocarbons served as control. The bacterial growth on petroleum hydrocarbons was assessed by measuring the bacterial cell population and gravimetric measurement of extracted residual crude oil remaining in the culture medium post the period of inoculation.

3.2.6 Determination of hydrocarbon biodegradability using a bioreactor

A bioreactor system was constructed which composed of a reservoir medium, magnetic stirrer-heater, and an air pump. The mode of operation of the system was continuous one while the magnetic stirrer heater kept the temperature of the medium reservoir constant at 30°C. The air pressure was left at a constant rate and the medium pump pressure was also kept at a constant flow rate. The set-up, which comprise of the flask, medium in reservoir bottle and tubing were sterilized by autoclaving at 121°C for 15 minutes. The hydrocarbon (hexadecane and crude oil) was aseptically added to the medium. The air out tube was

connected to an air-filter and tubing was placed in ethanol to prevent the spread any pathogenic bacteria.

i. Extraction of residual crude oil from the bioreactor set-up

Koma *et al.,* 2001 procedure was adopted for the extraction procedure. The chloroform – methanol (3:1) extraction method was used to extract the residual crude oil. Samples were aseptically obtained from the culture after every 48 h and centrifuged for 30 minutes at 5 500 rpm before extracting the aqueous crude oil suspension. The centrifuged samples were collected and the corresponding ratio of chloroform-methanol was added. The standard ratio was at 30 ml of chloroform-methanol to 100 ml of sample. The separation was carried out in a separating funnel and the extract was analyzed using gas chromatography (GC).

ii. Hydrocarbon analysis using gas chromatography.

The hydrocarbon in the mixture was extracted as described (**section 3.2.5-i)**. The GC (Thermo Finnigan, Trace) fitted with a flame ionization detector and capillary column type Rtx-5MS (30 x 0.25 mm). The GC was run on auto-sampling method and alkanes were detected by using a standard 0.1% Cyclohexane. The initial oven temperature was held at 50°C for 2 minutes while the injector and detector temperature were maintained at 270°C. The oven was programmed to rise from 50°C to 270°C at 10°C / min increment and held at 270°C for 15 mines. The injector was run on Split less Split less (SS) mode while 1 µl of the sample taken from the vial bottom was analyzed. Hydrogen flow was maintained at 35 ml/min, airflow at 350 ml/min, and makeup gas nitrogen at 30 ml/min while the carrier gas used was helium.

A standard calibration graph was plotted using the peak area obtained from gas chromatography (GC) of the hexadecane concentration in the range 0.025 – 2.0g /100ml analysis. The line of best fit was used to determine unknown concentrations of hexadecane. The increase in peak area was directly proportional to the concentration of hexadecane.

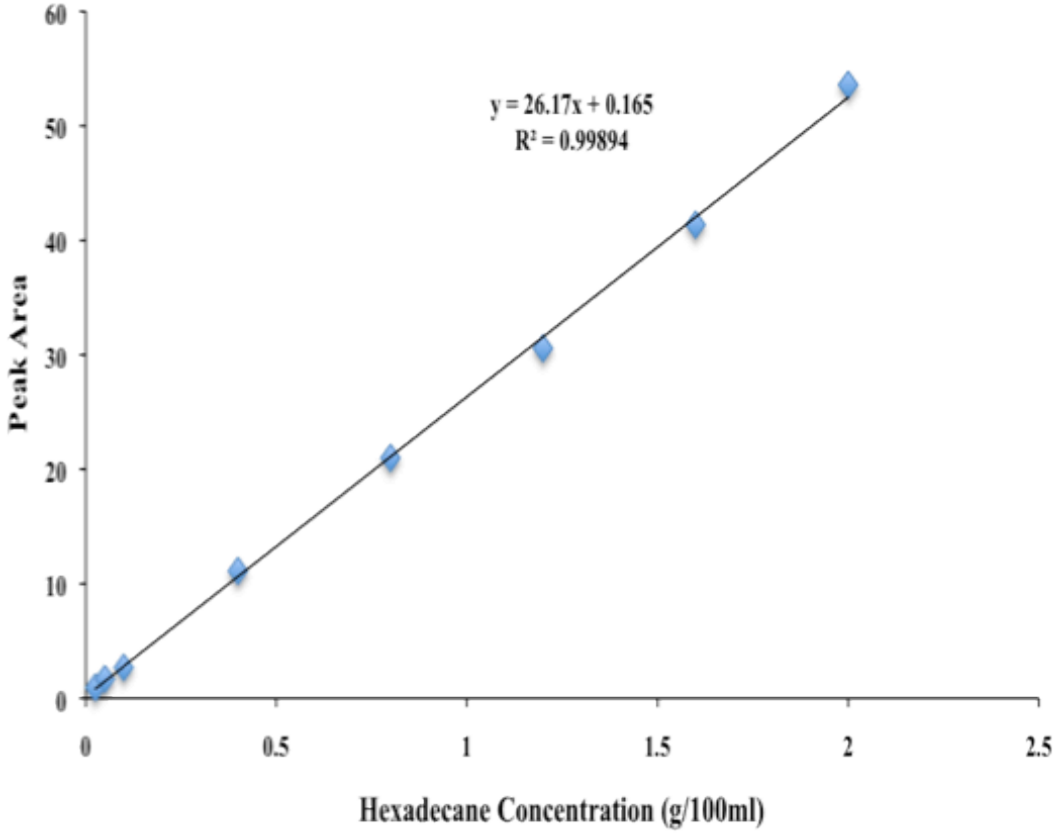

Figure 3.1 Hexadecane calibration graph using GC analysis. Note- peak area (mV*min)

3.3 RESULT:

3:3.1 Bacteria identification and screening for hydrocarbon degradation

The aim of this investigation was to screen the different bacterial isolates from University of Wolverhampton Culture Collection (UWCC) for hydrocarbon degradation at different temperatures and to identify the range and optimal temperature for the growth of these hydrocarbon degraders, which will be utilize in soil bioremediation investigation.

The agar plate method was used to screen a total of 21 bacterial isolates **(See Appendix: Table I.1)** for growth in the temperature range 25-50ºC using the general purpose medium TSA and BSMA plates (0.1%) with hexadecane and sodium benzoate as the hydrocarbon source. Amongst which three bacterial isolates (See Table 3.1) were selected as a result of their heavy growth level on the BSMA hydrocarbon plates and were further screened using the shake flask method.

Temperature was observed to influence the growth of the three bacteria selected on the hydrocarbon agar plates. *Pseudomonas aeruginosa* at 25ºC showed positive growth on both hexadecane and sodium benzoate, optimal growth was observed at 30ºC while no growth was obvious at 40ºC and 50ºC. *Acinetobacter sp* and *Rhodococcus sp* both exhibited a similar growth pattern except that at 37ºC, were *Pseudomonas aeruginosa* showed a heavy growth while they both showed positive growth on hexadecane but in the case of sodium benzoate they exhibited light growth at 37ºC while *Pseudomonas aeruginosa* showed a positive growth. The most suitable temperature selected based the three bacterial growth was 30ºC. This temperature (30ºC) was the incubation temperature employed for further investigation

Table 3.1 Selected screened bacteria for hydrocarbon degradation at varying temperatures.

Bacteria isolate	25ºC			30ºC			37ºC			40ºC			50ºC		
	TSA	HEX	SB	TSA	HEX	SB	TSA	HEX	SB	TSA	HEX	SB	TSA	HEX	SB
Rhodococcus sp	++	+	+	+++	+++	+	++	+	+/-	+/-	-	-	-	-	-
Pseudomonas aeruginosa	++	+	+	+++	+++	++	++	++	+	+/-	-	-	-	-	-
Acinetobacter sp	++	+	+	+++	+++	+	++	+	+/-	+/-	-	-	-	-	-

(+++) growth in clumps, (++) heavy growth, (+) positive growth, (+/-) light growth and (-) negative growth. TSA represent trypton soya agar, HEX stands for hexadecane and SB stands for sodium benzoate. Media was incubated in shake flasks for period of 48 h (for the TSA) and 144 h (for HEX and SB agar plates).

3:3.2 Effect of hydrocarbon concentrations on the growth of bacteria

The aim of this experiment was to assess the effect of different hydrocarbon concentrations on the growth of bacteria and establish the most effective concentration for the screened hydrocarbon degraders.

3.3.2.1: Effect of hexadecane concentrations on the growth of Pseudomonas aeruginosa using the bioscreen growth analyzer (BGA).

The growth dynamics of *P. aeruginosa* **(Figure 3.2)** were established by OD_{540nm} measurement using the BGA whereby BSM mineral media was supplemented with different concentration (0.5%, 1.0% and 2.0%) of hexadecane as the carbon source. Most growth of cells on the different concentrations of hexadecane was demonstrated within the first 24 h

period of incubation and followed by slower period of growth. Increase in the OD is an indication of growth of respiring cells, where hydrocarbon (hexadecane) was the sole carbon source as such implies the concentration of hexadecane decreased.

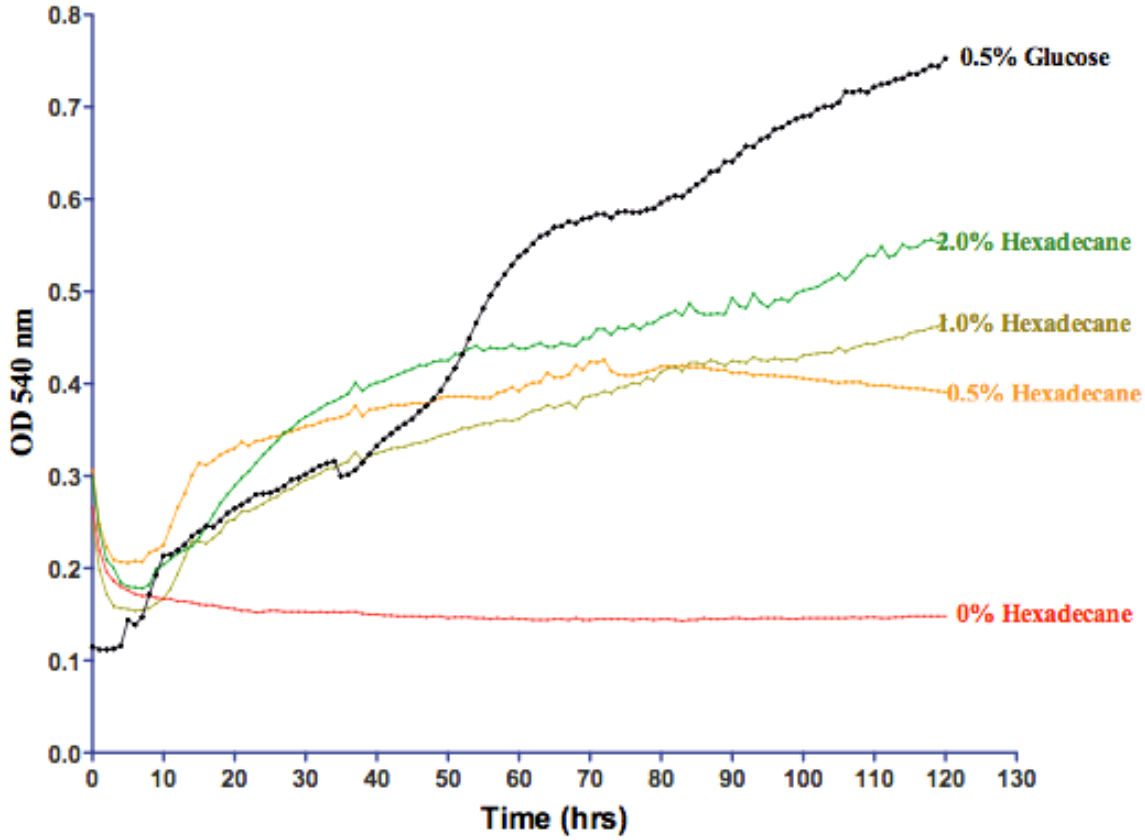

Figure 3.2 shows a typical graphical representative of the effect of Hexadecane concentrations on the growth of bacteria isolate *Pseudomonas aeruginosa* in BSM media at 30 °C by BGA

P. aeruginosa showed a lag phase in the different concentration of hexadecane **(Figure 3.2)**. The growth of the bacteria in 0.5% hexadecane concentration had a shorter lag and faster growth rate as compared to the 1.0% and 2.0% concentration. There is a difference in terms of the effect of the hexadecane concentrations and the mean generation time of the *P. aeruginosa* ($P < 0.05$). *P. aeruginosa* growth profile analysis shows that as the hexadecane concentration in the BSM growth media increases the mean generation time of the bacteria increased. The prompt stationary phase and subsequent decline in the growth pattern *Pseudomonas aeruginosa* in 0.5% hexadecane in salt media at 72 h of incubation is an indication of reduction in the carbon source utilized by the bacteria for energy and growth. However, in the

case of *P. aeruginosa* cells in 1.0% and 2.0% hexadecane in the growth media steady growth was still maintained which suggest that there were more carbon sources available in the media for the bacteria to make use of for its energy and growth allowing it to achieve a higher yield of cells.

Table 3.2 (a) Growth curve analysis of *Pseudomonas aeruginosa* isolates lag time, growth rate constant and mean generation time with different concentrations of hexadecane at 30 ºC using the BGA

Bacteria Isolate	**Hexadecane concentration (%)**	**Lag time (Hours)**	**Growth rate constant-μ ($Hours^{-1}$)**	**Mean generation (Hours)**
Pseudomonas aeruginosa	0.5	8.330 ± 0.420	0.042 ± 0.004	15.940 ± 0.762
	1.0	9.930 ± 0.120	0.036 ± 0.005	19.095 ± 1.035
	2.0	9.270 ± 0.460	0.034 ± 0.002	20.387 ± 0.763

Results represent the mean ± standard deviation of growth curve analysis of *Pseudomonas aeruginosa* on different concentration of hexadecane in replicated experiment (n=3)

The controls were set up during the investigation: OD_{540nm} measurement of only the mineral media salt (BSM), hexadecane and mineral salt media (BSM + HEX) and bacteria culture in mineral salt media (*Pseudomonas sp* + BSM). The OD_{540nm} readings from the control experiments did not show any form of growth in them as shown in **(Figure 3.2)** confirming that the change in OD in the presence of cells and hexadecane was due to bacterial growth.

3.3.2.2: Effect of hexadecane concentrations on the growth of Pseudomonas aeruginosa using the shake flask method (SFM).

The shake flask method was further used to establish the effect of concentrations (0.5-2.0%) of hexadecane the growth of *Pseudomonas aeruginosa* and to evaluate whether measured aeration might change the growth response to hydrocarbon concentrations. The poor aeration experienced while using the bioscreen growth analyzer influenced the need to use the shake flask method.

The starting inoculation size of *Pseudomonas aeruginosa* at day 0 incubation was 2.0×10^4 cfu/ml. This increased by two-fold population size at 24 h of inoculation in the media

containing different concentrations of hexadecane. The bacterial growth in 0.5% glucose BSM media was more than those on the three different hexadecane concentrations. However, 0.5% hexadecane showed greater level of growth as compared to 1.0% and 2.0% **(see figure 3.3)** but with no significant difference in each bacterial growth in the hexadecane BSM media which was confirmed further ($p>0.0001$).

At 96 h period of incubation, the bacterial cell population increased with 0.5% hexadecane still more as compared to the other hexadecane concentrations but subsequently declined at 144 h. However, in 1.0% and 2.0% hexadecane concentrations, *Pseudomonas aeruginosa* still maintained a steady increase in cell population. At 192 h, the bacterial cell population in 0.5% hexadecane had declined to 6.3×10^5 cfu/ml while that 1.0% and 2.0% hexadecane had increase to 4.0×10^8 cfu/ml and 4.1×10^9 cfu/ml respectively. The bacterial cells population in the growth media without any carbon source after 24 h began to steadily decline throughout the period of the experiment.

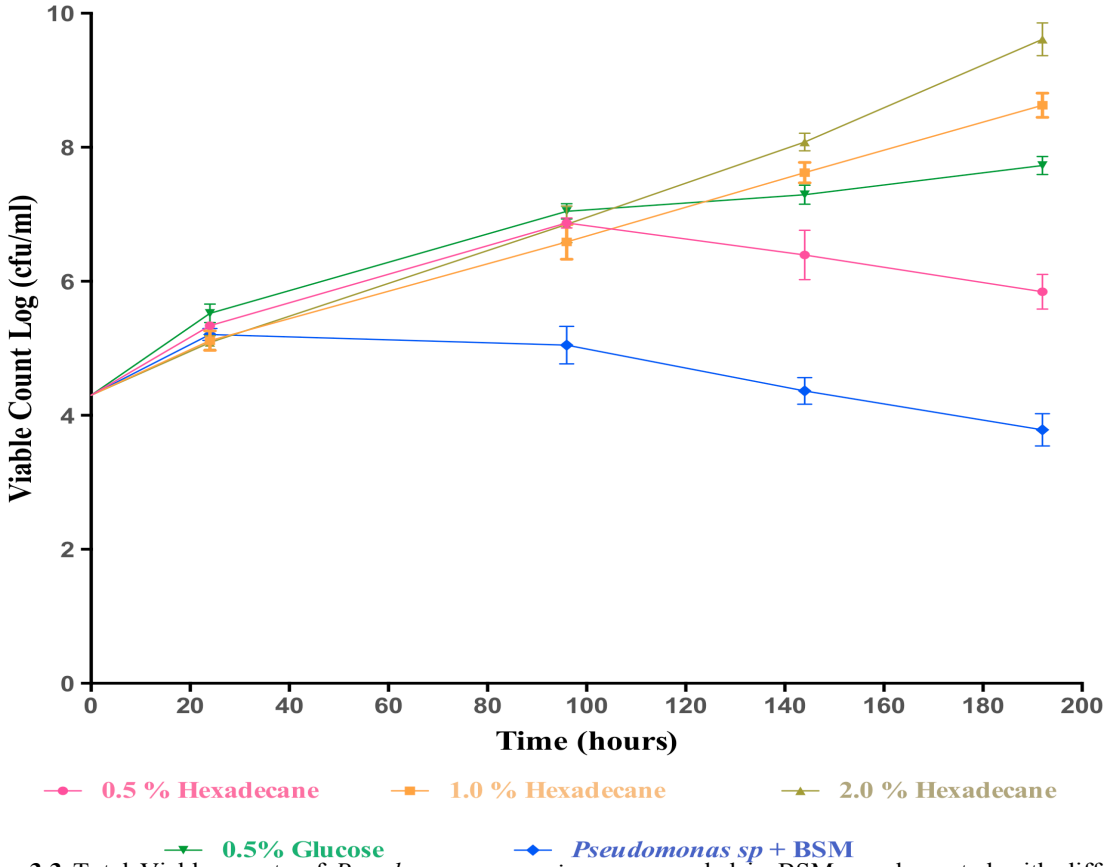

Figure 3.3 Total Viable counts of *Pseudomonas aeruginosa* suspended in BSM supplemented with different concentration of hexadecane at 30 °C -the error bar represents SD of replicated experiments (n=3)

The growth profile of *Pseudomonas aeruginosa* using the shake flask method presented in **Table 3.2 (b)** revealed a faster growth rate on the different hexadecane concentrations as compared to the bioscreen growth analyzer method. The reason for the estimating the bacteria growth rate for the shake flask was because unlike the bioscreen growth analyzer that had the bacteria growth monitored at specific lower interval (1 h) presented a more accurate growth curve (**Figure 3.2**), the bacterial growth from the shake flask had a wider interval of bacterial growth monitoring making it difficult to calculate the exert growth rate (**Figure 3.3**).

Table 3.2 (b) Growth curve analysis of *Pseudomonas aeruginosa* in different concentrations of hexadecane at 30 ºC using the shake flask methods

Bacteria Isolate	Hexadecane concentration (%)	Growth rate constant-μ ($Hours^{-1}$)	Mean generation (Hours)
Pseudomonas aeruginosa	0.5	0.085 ± 0.002	08.180 ± 0.772
	1.0	0.063 ± 0.001	11.011 ± 0.312
	2.0	0.052 ± 0.002	13.208 ± 0.513

Results represent the mean ± standard deviation of growth curve analysis of *Pseudomonas aeruginosa* on different concentration of hexadecane in replicated experiment (n=3)

3.3.2.3: Effect of hexadecane concentrations on the growth of Rhodococcus sp using the bioscreen growth analyzer (BGA).

The impact of hexadecane concentrations on the growth of *Rhodococcus sp* in a BSM media supplemented with hexadecane concentrations of 0.5%, 1.0% and 2.0% over a period of 120 hours at 30 °C was investigated. The bacteria showed a lag phase in growth within the first 5 h of inoculation on three of the hexadecane concentrations but gradually increased there after. Most growth of *Rhodococcus sp* on the different concentrations of hexadecane was demonstrated within the 20 h period of incubation. The bacteria showed a similar pattern of growth on the different hydrocarbon concentrations without significant difference ($p>0.0001$). However, after 40 h incubation period the growth of *Rhodococcus sp* on 0.5% hexadecane mineral salt media began to decline while those of 1.0% and 2.0% continued to grow. The decrease in OD can be attributed to non-availability of hexadecane the sole carbon sources for

it to utilize for energy and growth. Growth of *Rhodococcus sp* on 1.0% (w/v) of hexadecane showed the highest growth level based on the OD reading of about 0.52 as shown in **Figure 3.4.** The growth of the bacteria on 0.5% glucose was slow. The bacteria *Rhodococcus sp* showed a better growth rate in the BSM supplemented with hexadecane as compared with the 0.5% glucose.

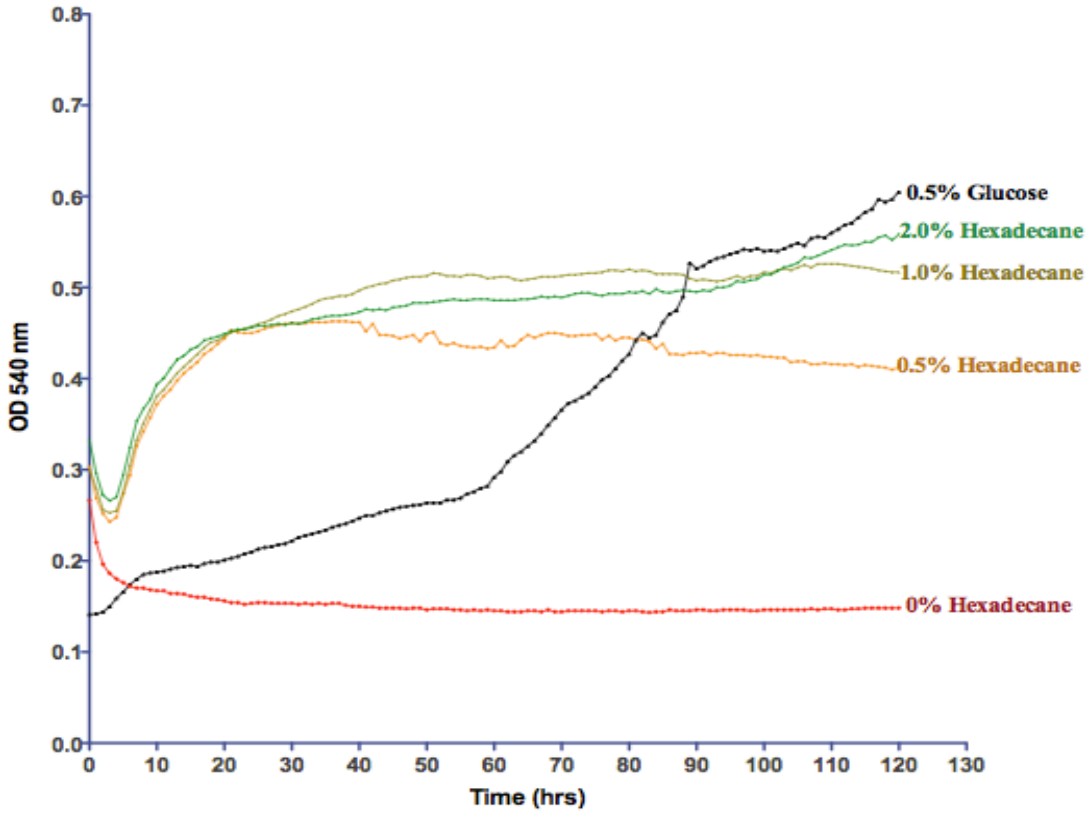

Figure 3.4 shows a typical graphical representative of the effect of Hexadecane concentrations (0.5-2.0%) in BSM media on the growth of bacteria isolate *Rhodococcus sp* at 30 °C by BGA

A summary of the growth curve analysis of *Rhodococcus sp* in terms of lag time, growth rate constant and mean generation time on different hexadecane concentration using the bioscreen growth analyzer in **Table 3.4 (a)** shows that there was no significant difference in observed of *Rhodococcus sp* in terms of the lag phase on the different hexadecane concentrations ($p<0.005$). Interestingly, the bacteria exhibited a faster doubling time (09.20 ± 2.77) h in the hydrocarbon with the highest concentration (2.0%). The mean generation time for

Rhodococcus sp on 0.5 and 1.0% hexadecane was 11.23 ± 0.72 h and 11.02 ± 3.12 h respectively.

Table 3.4 (a) Growth curve analysis of *Rhodococcus sp* isolates with different concentration of hexadecane at 30 ºC bioscreen growth analyzer

Bacteria Isolate	**Hexadecane concentration (%)**	**Lag time (Hours)**	**Growth rate constant-μ (Hours^{-1})**	**Mean generation (Hours)**
Rhodococcus spa	0.5 1.0 2.0	3.30 ± 0.44 3.23 ± 0.45 3.13 ± 0.29	0.06 ± 0.02 0.06 ± 0.01 0.08 ± 0.02	11.23 ± 0.72 11.02 ± 3.12 09.20 ± 2.77

Results represent the mean ± standard deviation of growth curve analysis of *Rhodococcus sp* on different concentration of hexadecane in replicated experiment (n=3)

The exposure of *Rhodococcus sp* to increasing hexadecane concentration does impact on its growth dynamics. As observed in the mean generation time of the bacterial cell **Table 3.4(a);** there is a significant difference in terms of the effect of the hexadecane concentrations on the mean generation time of the *Rhodococcus sp*, proven by the one-way ANOVA statistical analysis ($P<0.05$). It was observed from the growth profile analysis of *Rhodococcus sp* that as the hexadecane concentration in the BSM growth media increased the mean generation time of the bacteria decreased.

3.3.2.4: Effect of hexadecane concentrations on the growth of Rhodococcus sp using the shake flask method (SFM).

The aim of the experiment is to confirm the effect of hexadecane concentrations on the growth of *Rhodococcus sp* and establish the most suitable concentration for the optimal growth of the bacteria in a more oxygenated shake flask system.

Rhodococcus sp showed an increase in growth within the first 24 h of inoculation on 0.5 %, 1.0% and 2.0% hexadecane concentrations. There was no obvious lag phase experienced during this period with the shake flask **(see Figure 3.5)** as compared with the BGA method **(see Figure 3.4)**. However, the growth on 0.5% glucose was slightly lower than those of hydrocarbon concentration. At 96 h the bacterial still maintained a steady growth profile

showing no significant difference ($p>0.05$) in growth amongst each of the hydrocarbon concentration. The growth of the bacteria on 0.5% glucose was still low and significantly less than on 0.5% hexadecane concentration, which was also confirmed from the t' test ($p<0.05$). After 144 h of incubation, *Rhodococcus sp* gradually began to decline in growth on 0.5% hydrocarbon concentration through to the 192 h while those on 1.0 and 2.0% hexadecane concentration maintained a steady increase in growth. The decrease in the cell population of *Rhodococcus sp* on 0.5% hexadecane concentration of the growth media after the 144 h of incubation indicates decrease in the level of hexadecane. Interestingly, *Rhodococcus sp* growth declined from 24 h of inoculating in to the growth BSM media without carbon source. *Rhodococcus sp* isolates were able to make use of hexadecane as the carbon sources for growth and energy irrespective of the starting concentration.

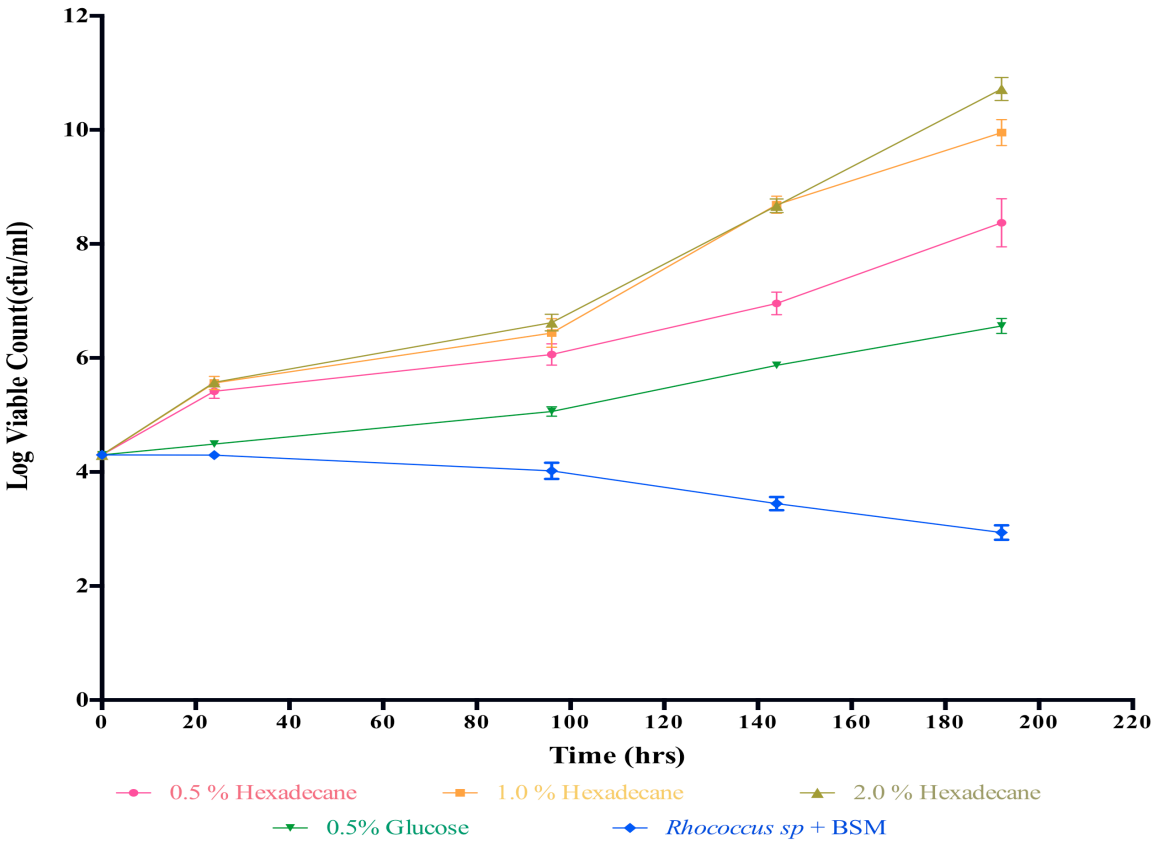

Figure 3.5 Total Viable counts of *Rhodococcus sp* suspended in BSM supplemented with different concentration of hexadecane at 30 °C - the error bar represents SD of replicated experiments (n=3)

An estimated summary of the growth rate of *Rhodococcus sp* on the different hexadecane concentrations using the shake flask shown in **Table 3.4 (b)** revealed that, the bacteria growth on 1.0% hexadecane concentration was faster than 0.5% and 2.0%. *Rhodococcus sp* also showed a faster growth rate in all the hexadecane concentrations in the shake flask compared to the bioscreen growth analyzer.

Table 3.4 (b) Growth curve analysis of *Rhodococcus sp* in different concentration of hexadecane at 30 ºC using the shake flask method

Bacteria Isolate	**Hexadecane concentration (%)**	**Growth rate constant-μ ($Hours^{-1}$)**	**Mean generation (Hours)**
Rhodococcus sp	0.5 1.0 2.0	0.11 ± 0.02 0.14 ± 0.01 0.13 ± 0.02	06.53 ± 0.12 05.11 ± 0.97 05.54 ± 0.47

Results represent the mean ± standard deviation of growth curve analysis of *Rhodococcus sp* on different concentration of hexadecane in replicated experiment (n=3)

3.3.2.5: Effect of hexadecane concentrations on the growth of Acinetobacter sp using the shake flask method (SFM).

This experiment aimed to determine the impact of different hexadecane concentrations on the growth of *Acinetobacter sp* and establish the most suitable concentration for the optimal growth of the bacteria in shake flasks.

The results in **(Figure 3.6)** showed that *Acinetobacter sp* exhibited an increase in cell population within the first 24 h of incubation without any obvious lag phase. This increase occurred with all the hexadecane concentrations in mineral media, which indicated that the hydrocarbon was utilized as the carbon source for energy and growth. The bacteria also showed higher growth on 0.5% glucose during the 24 h. At 92 h, the bacteria still maintained a steady increase growth pattern on all the hexadecane concentrations. At 144h, *Acinetobacter sp* cell population began to decline BSM supplemented with 0.5% hexadecane which probably indicated reduction of available carbon source for the bacteria to utilize but still

maintained growth on 1.0 and 2.0% hexadecane concentrations. The bacteria maintained a similar growth pattern on 196 h.

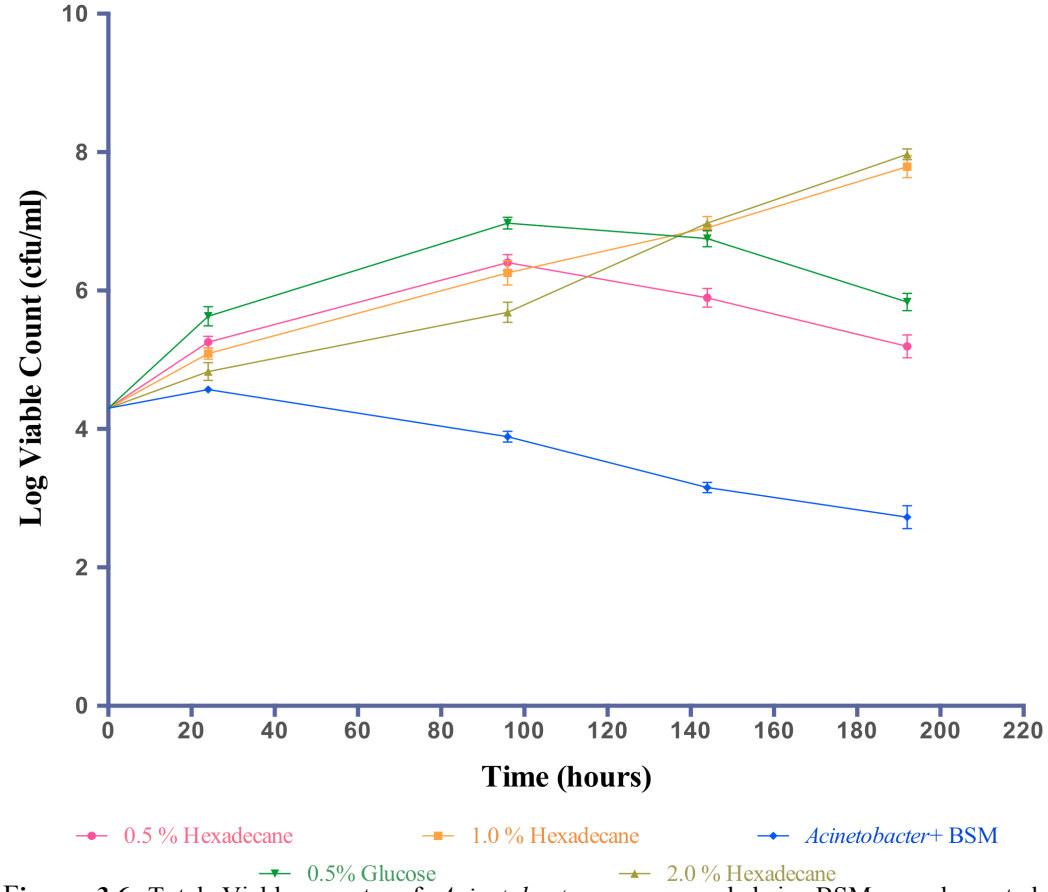

Figure 3.6 Total Viable counts of *Acinetobacter sp* suspended in BSM supplemented with different concentration of hexadecane at 30 °C - the error bar represents SD of replicated experiments (n=3)

An estimate summary of the growth rate of *Acinetobacter sp* on the different hexadecane concentrations using the shake flask (**Table 3.5)** showed bacteria growth on the different hexadecane concentration.

Table 3.5 Growth curve analysis of *Acinetobacter sp* in different concentration of hexadecane at 30 ºC using the shake flask method

Bacteria Isolate	**Hexadecane concentration (%)**	**Growth rate constant-μ (Hours^{-1})**	**Mean generation (Hours)**
Acinetobacter sp	0.5 1.0 2.0	0.05 ± 0.01 0.04 ± 0.03 0.04 ± 0.04	15.03 ± 0.72 16.22 ± 1.22 17.90 ± 0.97

Results represent the mean ± standard deviation of growth curve analysis of *Acinetobacter sp* on different concentration of hexadecane in replicated experiment (n=3)

3.3.2.6: The impact of sodium benzoate (SB) on the growth of Pseudomonas aeruginosa, Acinetobacter sp and Rhodococcus sp using the bioscreen growth analyzer.

The aim of this study was to determine the growth of *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* on sodium benzoate.

The growth dynamics **(Figure 3.7)** of *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* on 0.5% sodium benzoate were determined by measuring the OD_{540nm} in the bioscreen growth analyzer. The different bacterial isolates showed different growth rate in the media with varying lag phases **(Table 3.5).** *Pseudomonas aeruginosa* showed a lag phase for about 18.67±0.87 h before the sudden lengthy exponential phase that lasted for a period of 29 h, which was then followed by a stationary phase from the 49 h of incubation.

The *Pseudomonas sp* was observed to have a growth rate of 0.16 ± 0.03 h^{-1}. *Rhodococcus sp* demonstrated growth level in 0.5% sodium benzoate with a long lag phase of 22.87± 0.98 h. The *Rhodococcus sp* exhibited a growth rate of 0.11± 0.02 h^{-1} short exponential phase growth that lasted for a period of 27 h before the stationary phase of the bacterial growth. *Acinetobacter sp* had a longer lag phase with an exponential phase growth that lasted for 32 h. the bacterial showed a growth rate of 0.10±0.03 h^{-1}. The lag phases experienced in the different bacterial growth on the 0.5% could be attributed to the period of adaptation to the hydrocarbon and because of the poor aeration and agitation experienced in the bioscreen growth analyzer. A statistical comparism of the growth rates of the three bacteria on 0.5% sodium benzoate showed difference in growth ate 72 h of the investigation.

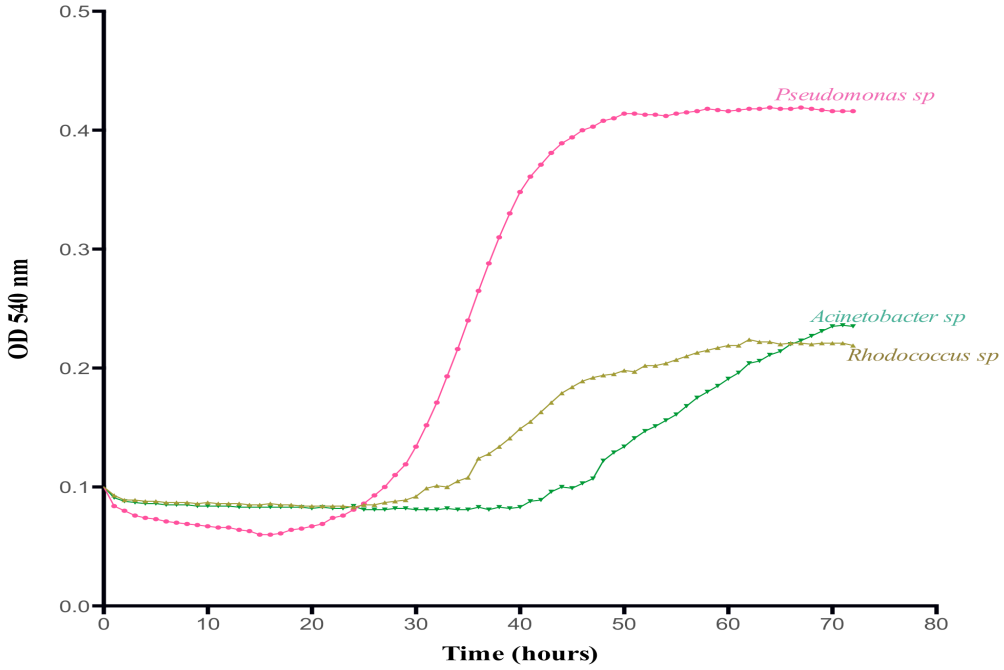

Figure 3.7 shows a typical growth of bacteria isolate *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* on 0.5% Sodium benzoate in BSM media at 30 °C by BGA

Table 3.6 Growth curve analysis of *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* on 0.5% sodium benzoate in BSM media at 30 °C

Bacteria Isolate	**Lag time (Hours)**	**Growth rate constant-μ ($Hours^{-1}$)**	**Mean generation (Hours)**
Pseudomonas aeruginosa	18.67 ± 0.87	0.16 ± 0.03	4.24 ± 0.87
Rhodococcus sp	22.87 ± 0.98	0.11± 0.02	6.03 ± 0.91
Acinetobacter sp	38.04 ± 1.09	0.10± 0.03	6.62 ± 0.45

Results represent the mean ± standard deviation of bacterial isolates growth curve analysis on 0.5% sodium benzoate in BSM media in replicated experiment (n=3)

3.3.3. The growth of bacteria on crude oil using the shake flask method.

The aim of this experiment was to establish the level of growth of *Pseudomonas aeruginosa, Acinetobacter sp*, *Rhodococcus sp* and an additional bacterial isolate (*Bacillus sp*) in crude oil and determine the extent of the hydrocarbon degradation. *Bacillus sp* was included in other to increase the number of bacterial cultures needed in the next phase of the investigation (soil bioremediation).

The bacterial isolates were pre-exposed to 0.5% crude oil 24 hrs prior to inoculation. The results of growth of the bacterial isolates in BSM supplemented with 1.0% petroleum crude

oil determined using the shake flask method are presented in **figure 3.8**. The growth media had a pH of 7.0 and the starting inoculum population of each of the bacteria was determined as 1.2 x 10^5 cfu/ml. All the bacterial strains except for *Bacillus sp* did not exhibit a lag phase during the first 24 h of inoculation. The individual bacteria maintained a slow but steady growth rate through to the 72 h period of culture except for the *Bacillus sp* where the cell population declined to 9.4x 10^3 cfu/ml at 168 h of incubation. However, *Pseudomonas aeruginosa, Acinetobacter sp*, *Rhodococcus sp* cells at 168 h increased in cell population to 9.8 x 10^5 cfu/ml, 8.5 x 10^5 cfu/ml and 1.3 x 10^6 cfu/ml respectively. The level of growth achieved by these bacteria at the 168 h period of investigation was poor due to the fact that the cells had not finished growing. All the bacterial strains except for *Bacillus sp* utilized the crude oil hydrocarbon as sole source of carbon and energy, which is evident from the increase in the total viable cell count after a period of 7 days as such, was excluded in the next phase of the investigation.

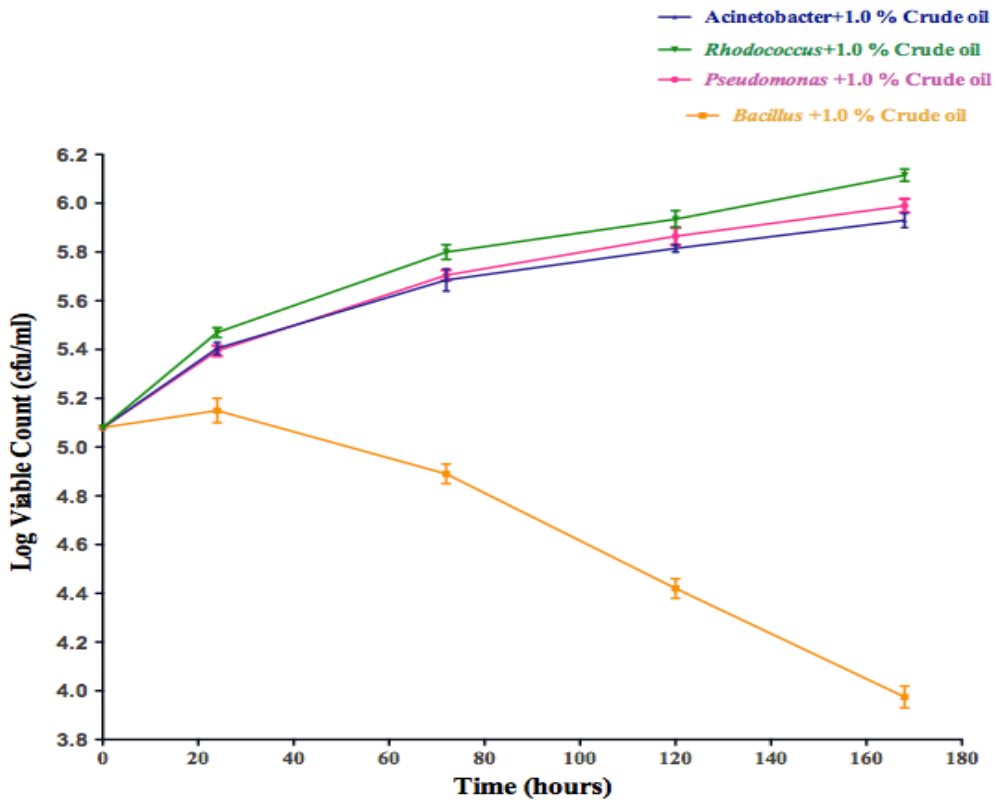

Figure 3.8 Shows the growth of *Pseudomonas aeruginosa, Bacillus sp, Acinetobacter sp* and *Rhodococcus sp* in terms of total viable count on 1.0 % crude oil supplemented in BSM at 30 °C for a period of 7 days- the error bar represents SD of replicated experiments (n=3)

3.3.4. The growth of bacteria on crude oil using the bioreactor

The shake flask experiment for growth on the crude oil indicated slow growth and may be due inadequate agitation to advere adequate mixing of the crude oil in the BSM. Consequently, a bioreactor with mechanical stirer was used to determine the growth of the selected bacterial mixed culture on crude oil.

A mixed culture of the three bacterial isolates (*Pseudomonas aeruginosa, Acinetobacter sp*, *Rhodococcus sp*) pre-exposed to 0.5% crude oil 24 h prior to inoculation was formulated by utilizing equal individual bacterial cell population. The growth profile of the bacterial mixed culture was determined by the total viable count and pH **(Figure 3.9).** The growth media had an initial pH of 7.0 and an initial bacterial mixed culture inoculum size of 1.2 x 10^5 cfu/ml. After 24 h period of incubation, the cell population of the mixed culture increased to 2.5 x 10^6 cfu/ml. The steady growth continued till the 72 h with a cell population size of 1.0 x 10^8 cfu/ml. After 120 h, the bacterial consortium experienced a stationary phase growth rate from 1.0 x 10^8 cfu/ml to 1.6 x 10^8 cfu/ml at the 7th day's incubation. The pH of the content in the bioreactor monitored showed gradual decreases in value to 6.4.

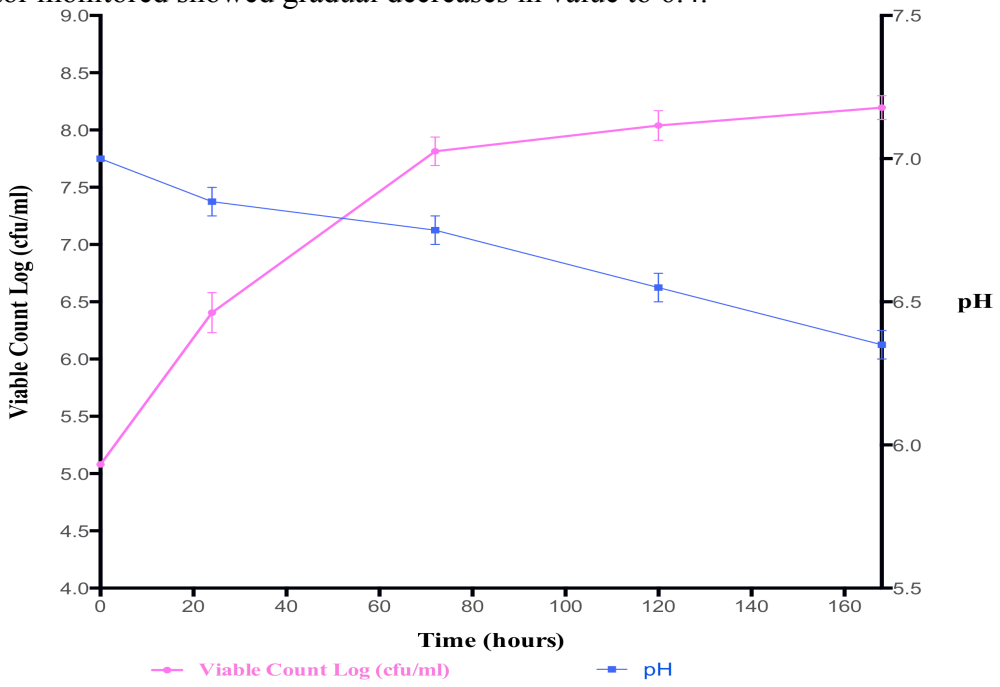

Figure 3.9 Shows the growth of a mixed culture of: *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* in BSM supplemented with 1.0 % crude oil supplemented in for a period of 7 days in a Bioreactor- the error bar represents SD of replicated experiments (n=3)

3.4 DISCUSSION

The screened hydrocarbon degraders **(Table 3.1)** from the UWCC at varying temperatures showed the influence of temperature on bacterial growth on hydrocarbons **Appendix I- Table I.1**. Temperature controls the bioavailability of low-solubility hydrocarbon and hence the nature and the extent of microbial metabolism (Margesin and Schinner, 2001). The optimal temperature for the selected bacteria was 30ºC. Si-Zhong *et al.* (2009) reported that the optimum temperature for biodegradation is usually 15-30 ºC for aerobic processes and 25-35 ºC for anaerobic processes. The ambient environment in the Niger Delta has a daily mean minimum and maximum temperature of 23 ^{o}C and 31.5 ^{o}C respectively (Ayotamuna *et al.*, 2006). It implies that these screened bacterial would be able to effectively survive the Niger Delta soil environment.

Amongst the further screening and bacterial growth method used to evaluate bacterial growth on hydrocarbon, the bioscreen growth analyzer method was identified to have some shortcomings which included: poor agitation of the culture and growth media, poor aeration-oxygen distribution in the system, inability to determine the cell count at interval with assumption that optical density is totally due to cell numbers and proportionally. The shake flask method employed was to resolve these shortcomings identified. Hydrocarbon degradation is a highly oxidative process in which molecular oxygen is essential (Sharma and Pant, 2001). Therefore for maximal bacterial growth and degradation effective aeration and agitation is required, thus the choice of the shake flask method for this experiment. The effect of aeration was evident in this investigation from the enhanced rate of growth effected by the shake flask culture system. Aeration conditions are considered essential for the growth for microbial growth and extensive degradation of crude oil in the environment (Abu and Dike, 2008)

Hydrocarbon concentration plays a significant role in its bioremediation (Del Arco and de Franca, 2000). This investigation of the effect of hexadecane concentrations on bacteria has revealed significant results with respect to individual bacterial growth. Increase in the concentration of hexadecane in the BSM affected a significant influence on the growth rate of *Pseudomonas sp* and was reflected in the lag time of the bacteria cell as shown (**Table 3.3)**. The growth rate of *Pseudomonas sp* using the bioscreen growth analyzer on 0.5, 1.0 and 2.0% hexadecane were observed as 0.042±0.004 h^{-1}, 0.036±0.005 h^{-1} and 0.034±0.002 h^{-1} respectively but on the shake flask was estimated as 0.085±0.002 h^{-1} , 0.063±0.001 h^{-1} and 0.052±0.002 h^{-1} respectively. Noordman *et al.,* 2002 recorded a growth rate of 0.02h^{-1} for *Pseudomonas aeruginosa* in 0.004-0.3% (v/v) hexadecane using a biosurfactant (rhamnolipid). The utilization of rhamnolipid would have enhanced the emulsification of the hexadecane which made it readily available for the bacterial utilization un-like in this investigation whereby biosufactant was not pre-added to the experimental design. Increase in hexadecane concentration in the BSM was observed to increase the doubling time of *Pseudomonas aeruginosa*. The *Pseudomonas sp* grew more rapid on 0.5% glucose than on the higher hydrocarbon concentrations. The rapid growth on the glucose could be attributed the simple nature of substrate and its easy conversion by the *Pseudomonas sp* for growth and energy as described in the metabolic pathway **(Chapter 1)**. Barnell and Conway, 1990 reviewed that the bacterial has genes that are switched on for glucose metabolism. However, the bacteria needed a more complex metabolic pathway to utilize hexadecane for growth e.g. for *Pseudomonas aeruginosa* (Das and Chandran, 2010) while *Rhodococcus sp* utilize the preformed fatty acids derived from the mono-terminal oxidation of the alkane for the triacylglycerol biosynthesis Alvarez *et al*.,(2013).

The growth dynamic of *Rhodococcus sp* on the different hexadecane concentrations and 0.5% glucose carbon sources **(see figure 3.4)** was completely different from that shown by

Pseudomonas sp. Rhodococcus sp showed a more rapid growth on hexadecane concentrations as compared to 0.5% glucose. This is in line with the observation of Whyte *et al*. (1999) who examining the physiological adaptation of *Rhodococcus sp* reported that its cell surface hydrophobicity affected it subsequently rate of growth. They found that it was able to mineralize alkane faster after growth on diesel fuel or hexadecane than those cells grown on glucose-acetate. *Rhodococcus sp* has been reported to posses the ability to degrade a large number of organic compounds including the more recalcitrant ones because it possesses a wide and diverse range of catabolic genes that can stand harsh conditions. (Alvarez, 2010). Alvarez *et al*., (2013) further provided an insight into the differences in growth pattern of *Rhodococcus sp* on the carbon source from glucose and hexadecane. They reported that when the bacterium is grown on glucose, the substrate has to be degraded to acetyl CoA a precursor for fatty acid biosynthesis. In contrast to when cultivation on hexadecane, the bacterial cells utilize the preformed fatty acids derived from the mono-terminal oxidation of the alkane for the triacylgycerols biosynthesis. The different metabolic pathway helps *Rhodococcus* to conserve metabolic useful energy during catabolism of carbons, thus, a part of the resulting energy used for growth and division. The growth rate constant for *Rhodococcus sp* on 0.5, 1.0 and 2.0% concentrations of hexadecane using the bioscreen growth analyzer was 0.06 ± 0.02 h^{-1}, 0.06± 0.01 h^{-1} and 0.08 ± 0.02 h^{-1} respectively, while on the shake flask had calculated growth rate of 0.11 ± 0.02 h^{-1}, 0.14± 0.01 h^{-1} and 0.13 ± 0.02 h^{-1} respectively. This showed a more rapid growth of the bacterial using the shake flask. *Acinetobacter sp* was observed to show growth in all the hexadecane concentrations and 0.5% glucose.

Sun *et al.,* (2012) confirmed the growth *Acinetobacter sp* in n-hexadecane as it is detected to possess alkane monooxygenase gene that initiate oxidative degradation of this hydrocarbon. Dehghani *et al.* (2013) used the shake flask method to establish the degradation of n-hexadecane by *Acinetobacter radionesistens.* The increase in the bacterial cell population in

the different concentration of the hydrocarbon is an indication of the bacteria making use of hexadecane as the sole carbon source thus implying that the concentration of hydrocarbon was decreasing. Abdul-Megeed *et al.,* (2010) obtained similar results when degrading hexadecane with *Pseudomonas sp* and *Rhodococcus sp*. They observed a decline in the hexadecane concentration as the bacterial population increased.

The growth profile of the three bacterial isolates on 0.5% sodium benzoate was analyzed in terms of their lag phase, growth rate and doubling time (see Table 3.5) showed some differences in their adaptation and growth. *Pseudomonas aeruginosa* had a growth rate of $0.16 \pm 0.03\ h^{-1}$ on the 0.5% sodium benzoate, which was lower than the findings of Loh and Chua, (2002) when they experimented with *Pseudomonas putida*. They recorded a growth rate of $0.66\ h^{-1}$ on 0.2% sodium benzoate but used a more enriched minerals salt media (by adding phosphate buffer) than the one used in this investigation. *Rhodococcus sp* demonstrated growth rate and doubling time of $0.11 \pm 0.02\ h^{-1}$ and 6.03±0.03 h respectively on 0.5% sodium benzoate. This result was slightly higher than the findings of Choi *et al*., (2007) that reported a doubling time of 4.2±0.11 h when they studied the preference of utilization of benzoate using a *Rhodococcus sp* strain DK_{17}. The method of treatment of *Rhodococcus sp* during growth was different on 5mM (0.07% w/v) benzoate and higher agitation was used (180 rpm). The three bacteria from the investigation conducted so far have shown faster adaptation and growth in straight chain hexadecane as compared with sodium benzoate.

The bacterial cultures: *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* showed slow level of growth on 1.0% crude oil using the shake flask method while *Bacillus sp* was unable to grow (**Figure 3.8 & 3.9)**. The slow growth experienced by these bacteria may be due inadequate agitation in mixing of the crude oil in the BSM. Sham and Pant (2001) reported that oxygen is needed for hydrocarbon degradation and that agitation and aeration are required for maximal degradation of crude oil. They observed that as the agitation speed

increases, oxygen transfer rate incresased resulting to higher degradation of the crude oil by *Rhodococcus sp.* This was observed in this invesitigation when the bioreactor with magnetic stirrer was used to enhance agitation of the crude oil in BSM during the mixed culture degradation. The growth profile of the mixed culture of *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* showed an increase in the cell population from 1.2 x 10^5 cfu/ml to 1.6 x 10^8 cfu/ml after a period of 7 days incubation while the individual bacterial isolate had a less growth. A mixed culture of bacteria consortium is more effective than using a single bacteria culture. Westlakes (1982) reported that no single microbial species has the enzymatic ability to metabolize more than two or three classes of compound typically found in crude oil. The utilization of the crude oil by the bacterial consortium as the sole carbon sources and energy for growth is responsible for the cell increase while the acidic metabolic products may be responsible for the decrease in the pH of the growth media **(see Figure 3.9)**.

3.5 INTERIM CONCLUSIONS:

The following conclusions were recommended from the investigation:

- The maximal optimal temperature for the growth of the selected bacterial (*Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp)* on hexadecane and sodium benzoate was observed as 30 ^oC.
- *Rhodococcus sp* showed a more rapid growth on basal salt media (BSM) supplemented with hexadecane concentrations (0.5% -2.0 %) as compared to BSM supplemented with 0.5% glucose while *Pseudomonas aeruginosa* and *Acinetobacter sp* showed a more rapid growth on BSM supplemented with glucose compared to the hexadecane.
- The shake flask method was observed to be more effective for bacterial growth compared to the bioscreen growth analyzer.

- Aeration and agitation was observed to impact the level of bacterial growth, thus influenced bioremediation of petroleum hydrocarbon.
- The mixed bacterial consortium was observed to be more effective for bioremediation of petroleum crude oil as such the mixed culture of *Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp* is recommended for the soil phase bioremediation investigation

CHAPTER 4

SOIL CHARACTERIZATION, ANALYSIS AND FORMULATION – NIGER DELTA SOIL

4.1 INTRODUCTION

The aim of the soil characterization and analysis was to formulate a soil composition similar to that of the Niger Delta and to provide an understanding of the influence of the bacterial consortium and zeolite on bioremediation. In other to evaluate oil bioremediation in soil environments and the difficulty in obtaining soil samples from the Niger Delta region it was necessary to model the soil in the laboratory. After a thorough literature search of the Niger Delta soils (Akpokedje, 1987; Iwegbue *et al*., 2006) and Hilton soil (Fullen *et al*., 2006), the Hilton soil from East Shropshire, UK **(see Figure 4.1)** was used to model the Niger Delta soil due to similar characteristics in mineralogy and particle size distribution.

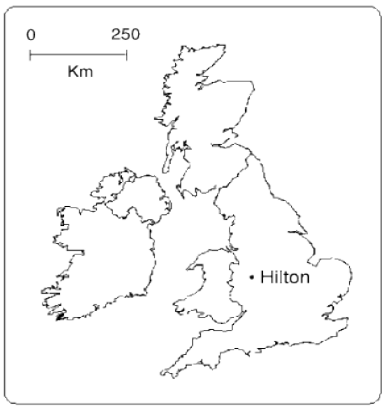

Figure 4.1 Location of the Hilton Experimental Site, east Shropshire, UK
(Source: Fullen *et al*., 2006)

The soil composition of the Niger Delta **(Chapter 1)** revealed that the region is underlined by different superficial soil deposit which varied widely from sandy to predominantly silty loam, sandy clay clayey/silty and loam texture Effiong and Ayolagha (2010). The Hilton soil was described by Fullen *et al*. (2006) to be made up of sandy, silty and clay soil. The Hilton soil was characterized and separated based on the particle size and then restructured to model that of Niger Delta soil. The zeolite used during this investigation was a sedimentary deposit

zeolite supplied from Hector, California USA and identified as clinoptilolite from the x-ray diffraction (XRD) analysis.

4.2 METHODS

4.2.1 Physical methods

4.2.1.1 Field collection

Soil samples were collected by hand auger from 3 different locations at a depth of 5-20 cm at the Hilton experimental site in East Shropshire, UK. Some were collected close to the flowing streamside while others from vegetation area by first taken off the debris and dead leaves. These soil samples were transported to the laboratory in a clean sterile container.

4.2.1.2 Soil preparation

The soils were homogenised and dried at 40 °C mixed for a period of 48 hours. The dried soil samples were disaggregated lightly by pestle and mortar prior to particle size analysis. The dried soil sample were passed through an arranged stack of six different mesh sizes in the following order 2.0 mm, 1.0 mm, 0.5 mm, 0.25 mm, 0.125 mm and 0.063 mm, using the Fritsch Vibratory Sieve Shaker for 15 minutes. The bottom pan collected particles < 0.063 mm in size. The sieved soils were properly labelled and stored for further experimentation.

4.2.3 Soil physico-chemical analysis

4.2.3.1 Soil moisture content

The moisture content of the soil was determined based on Gogoi *et al.* (2003) methods. 50 g of the dried soil samples was kept in the desiccators and the weight determined. The samples were then crushed in a mortar, sieved, re-dried and weighed.

$$\text{Moisture Content} = \frac{Mo - Md}{Mo} \times 100\% \qquad \text{Equation 4.1}$$

Where M_O = Mass of original soil sample

M_D = Mass of dried soil sample

4.2.3.2 Soil pH

The method for soil pH determination was based on Henning (2004) method where by 5 g of the crushed dried soil samples was placed in a vial and thoroughly mixed with 5 ml of deionized water. The solution was left for about 1-2 h with occasional stirring. The Corning pH meter 240 probe was inserted for 5 min and reading taken.

4.2.3.3 Soil total organic content

The soil organic matter was determined by the methodology of Ball (1964) and Nelson and Sommersr (1982); 1 g of soil sample was placed in a crucible and weighted accurately and transfers into an electric furnace and ignited at 375 ^{o}C for 5 hours. The crucible was removed, allowed to cool and re-weighed. The difference in the dry state yields the organic content.

$$\text{Total Organic Carbon} = \frac{Mo - Mi}{Mo} \times 100\% \qquad \text{Equation 4.2}$$

Where, M_O = Mass of original sample

M_i = Mass of sample after incineration

4.2.4 Soil geochemical properties analysis

4.2.4.1 Total elemental concentration

The x-ray fluorescence (XRF) Spectro Xepos Machine was employed to determine the total elemental concentration. The soil sample was grinded into powdered then 0.4 g of the powder soil samples was introduced into an open XRF tube which was covered with a transparent fine film and inserted into the XRF machine. A paladium x-ray source was used as the primary radation while helium was used as the air in the tube for the interaction. The analysis was conducted for 2 h and the total elemental concentration of the soil samples determined.

4.2.4.2 Mineralogical analysis

X-ray diffraction (XRD) of the soil was carried out using PW1729 x-ray generator Philips with copper x-ray sources for the mineralogical composition to the soils samples. The X- pert plus software was used to identify the mineral composition of the soil samples.

The XRD analysis involved four stages as represented in the flow chart in Figure 4.2

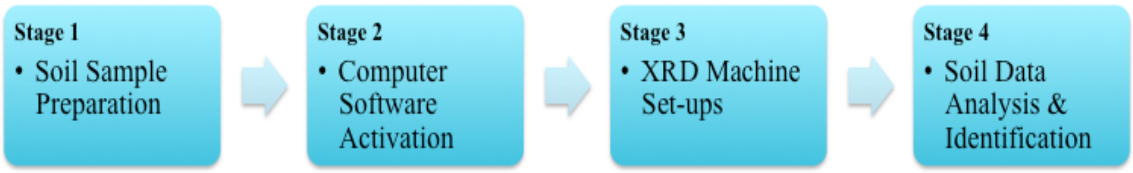

Figure 4.2 schematic illustrations of the processes involved in XRD

The soil sample was mounted onto a glass slide using two drops of ethanol onto the soil on the glass slide and uniformly slides through with another glass slide. The glass slide with the sample was secured on the x-ray in a central platform. The computer software was activated and the generator was set-up by slowly adjusting voltage knobs 5kV step to 40 kV followed by slowly increasing the current dial to 30 mA. The soil sample was allowed to run for 3 h while the computer was allowed to process the data. The analyzed soil samples were removed after closing the x-ray shutter and data retrieved, and the X-pert plus software was used for the identifying the mineralogy.

4.2.4 Soil formulation

The soil used for the bioremediation process was formulated according to Akpokodje, (1987) to represent the soil particle characteristics of the Niger Delta region of Nigeria. The Niger Delta soils are made up of different soil particle sizes but they are majorly composed of sandy, silty and clay soil Iwegbue *et al.,* (2006). The following soil particle sizes (1.0 mm, 0.5 mm, 0.25 mm, 0.125 mm) graded were equally combined together to formulate the sandy soil. The sandy soil was formulated by mixing together 500 g of each of the different particle sizes, thus producing sandy soil particle sizes (0.25-1.00 mm). The < 0.063 mm particles size was used to formulate the clay/silt soil used for the investigation. The soils were maintained within the temperature range of 25-30 ^{o}C in sterile bags to mimic the Niger Delta environment.

4.2.4 Scanning Electronic Microscopy (SEM)

The soil samples were freeze-dried for a period of 48 hours using the Edwards Freeze Dryer Modulyo. Individual freeze-dried samples were mounted on separate carbon metal conductor. They were then splutter-coated with gold using the Emscope SC 500. The coated samples were later examined using the Zeisis EVO 50 (Oxford Instrument, INCA wave model) scanning electron microscope at different working distances and magnification.

4.3. RESULTS

4.3.1 Soil physico-chemical analysis

The soil physicochemical properties are determined, sieved and graded into standard grain sizes of within the range (0.063 – 2.000) mm **(Appendix II Table I1-1b)**. The pH of soil particle size < 0.063mm was 6.73 ± 0.2 and subsequent soil particle sizes were observed to slightly decrease as the particle size increased except for 0.250 – 0.500mm that had a pH of 6.67 ± 0.3. However, the percentage total organic carbon in the particle size < 0.063 was observed to be 26.4 ± 0.3%, which was higher than the other graded particle sizes. The total organic carbon, nitrogen and phosphate in the different graded soil particle sizes was observed to decreased with increase in the soil particle sizes.

4.3.2 Soil geochemical analysis

4.3.2.1 X-ray fluorescence (XRF) analysis of soil samples

The XRF results of the soil samples are given in **Appendix II Table II-2** and revealed > 50 different elements. The major elements are sodium, magnesium, aluminium, potassium, iron and silicon as shown in Figure 4.3. Trace elements (Cr, Ni, Ba, Nb, Zr, Y, Sr, Rb, Co) were also identified in concentrations between 0.001% and <0.00009%.

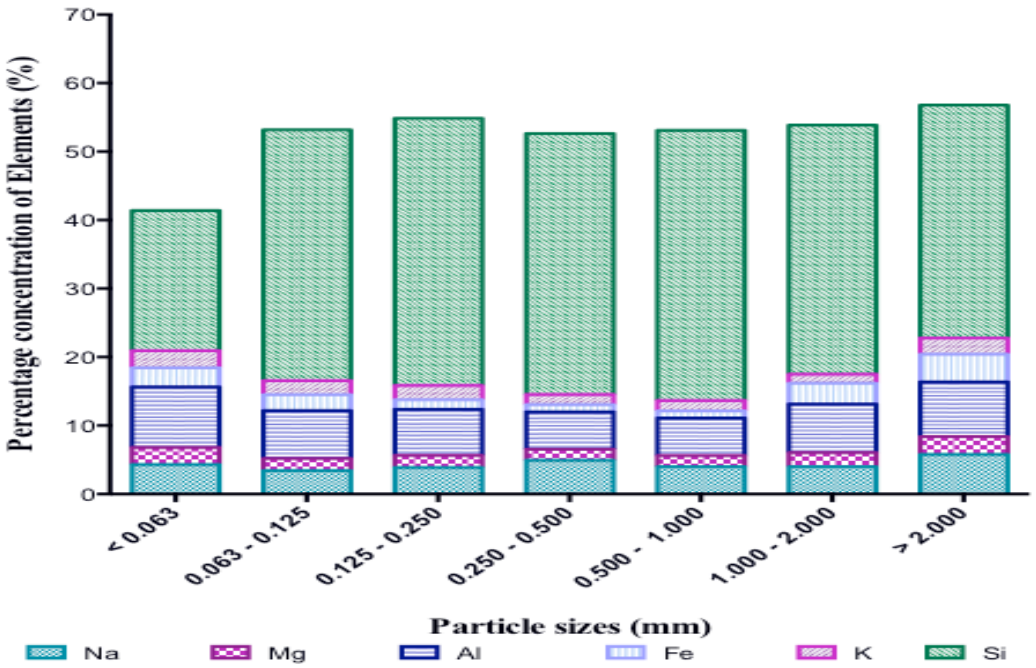

Figure 4.3 shows the elemental composition from the XRF analysis of the graded soil samples from the Hilton used in modelling the Niger Delta soil. Only elements whose percent concentration were greater than one are included

4.3.2.2 X-ray diffraction (XRD) analyses of soil sample

The XRD analysis of the soil sample revealed that quartz (SiO_2) is the predominant mineral present as shown in **Appendix II-Table II. 1a.** Kaolinite, illite and smectite were also present. This indicates similar mineralogical composition to soils in the Niger Delta. The spectroscopy of the graded soil particle sizes as shown Figure 4.4(a-f) reveal similar peak sizes indicating similarity in the particle sizes. The single high peak sizes found in the six graphical representatives are a clear indication of the presence of quartz (SiO_2) in the soil samples. The XRF result in Figure 4.3 earlier reviewed the high percentage of elemental concentration of silicon. The XRD results showed a high score match for quart-SiO_2. The smaller peak sizes are an indication of other minerals such as kaolinite, illite, smectite, which can be confirmed from the mineralogy in **Appendix II-Table II. 1a**, which are similar to that found in the Niger Delta soil.

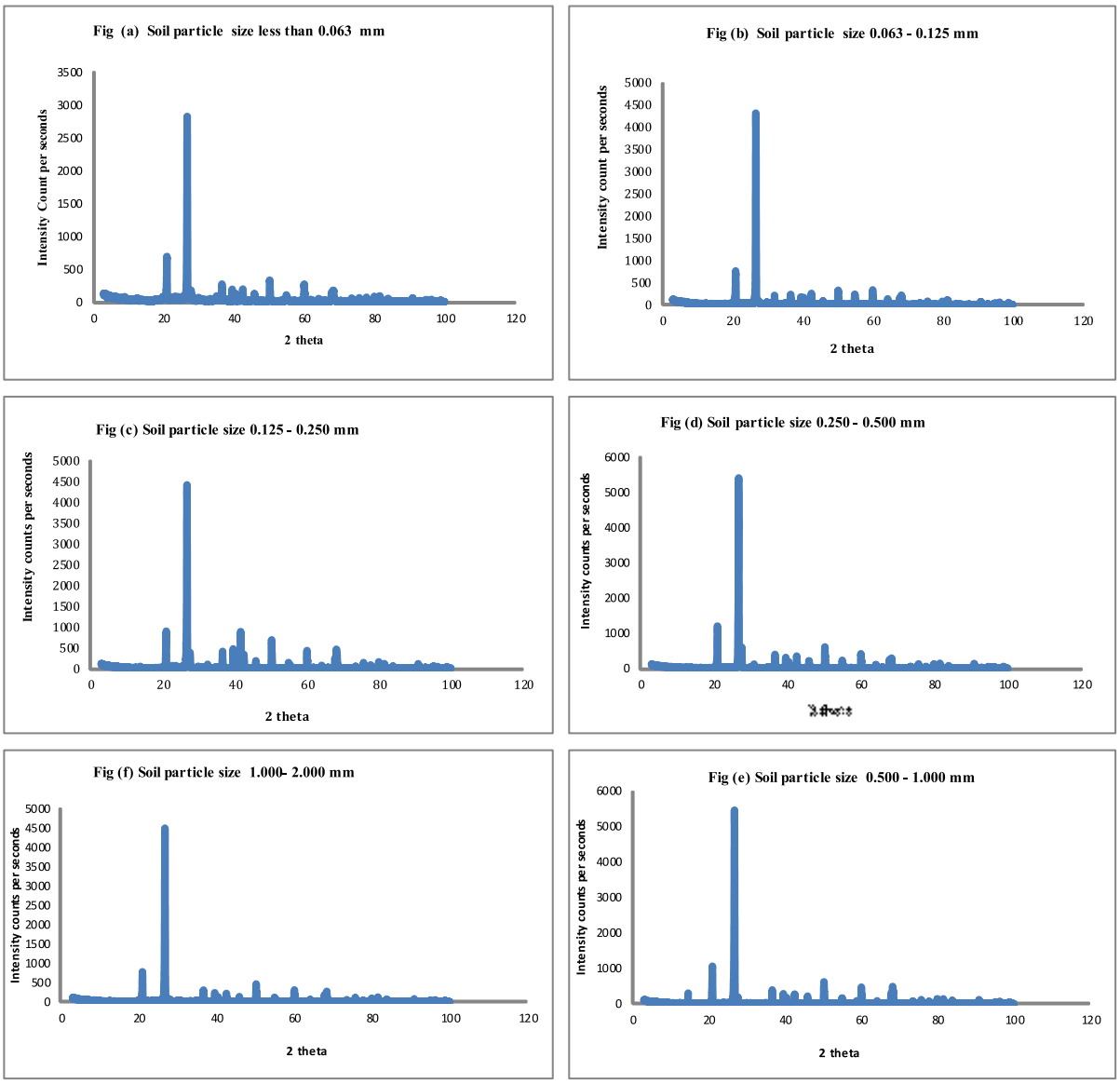

Figure 4.4(a-f) XRD Spectroscopy of the characterized Soil particle sizes of the Hilton soil used to model the soil of Niger Delta

4.3.3. Physiochemical analyses of the formulated silt-clay and sandy soil

The physiochemical analyses of the formulated silty-clay and sandy soils are shown in Table 3.2. The pH of the silty-clay soil was observed as 6.73 ± 0.14 while that of the sandy soil 6.45 ± 0.13 which indicates a slightly acidic soil. The organic carbon content in the silty-clay soil was determined from **Equation 4.2** as 26.4 ± 0.3% which was higher than the sandy soil with

total organic carbon of 19.4 ± 0.2%. The nitrogen and phosphate level in the formulated clay and sandy soil were slightly low as shown in Table 3.1.

Table 3.1 Profile description and characterization of the model Niger Delta soils

Physical appearance	**Physiochemical properties**	
Particle name	Silty -clay	Fine sandy
Particle sizes (mm)	< 0.063	0.25-1.00
Clay (%)	50	0
Silt (%)	50	0
Fine sand (%)	0	100
P^H	6.73 ± 0.14	6.45 ± 0.13
% Moisture by mass	25.4 ± 0.2	22.6 ± 0.2
Available PO_4 (ppm)	1.60 ± 0.12	1.30 ± 0.03
Nitrogen (%)	0.17 ± 0.01	0.14 ± 0.04
Organic Carbon (%)	26.4 ± 0.3	19.4 ± 0.4

Results represent the mean±standard deviation of three replicates

4.3.4. XRF and XRD analyses of the formulated silt-clay and sandy soil

The XRD results of the modelled clay and sandy soil see Table 3.2 showed the presence of higher level of quartz in the sandy soil as compared to the silt-clay. The low quartz level in the clay soil is as a result of the silty nature of the clay formulated based on the particle sizes composition. The XRD analysis of both soils reveal quart-SiO_2 to be predominant mineral present. The single high peak at angle 26 in the spectroscopy of the soil is an indication of the amount of quartz in the clay as in **Figure 4.5** and sandy soil as in **Figure 4.6** and is supported by XRF analyses showing high silicon content as shown in **Appendix II Table II. 2**. The smaller peaks are indication of kaolinite, illite and smectite, which can be confirm from the mineralogical composition of the soil shown in Table 3.2. Chlorite was observed to be present in low amounts in both soil samples as shown in **Appendix II Table II. 2.**

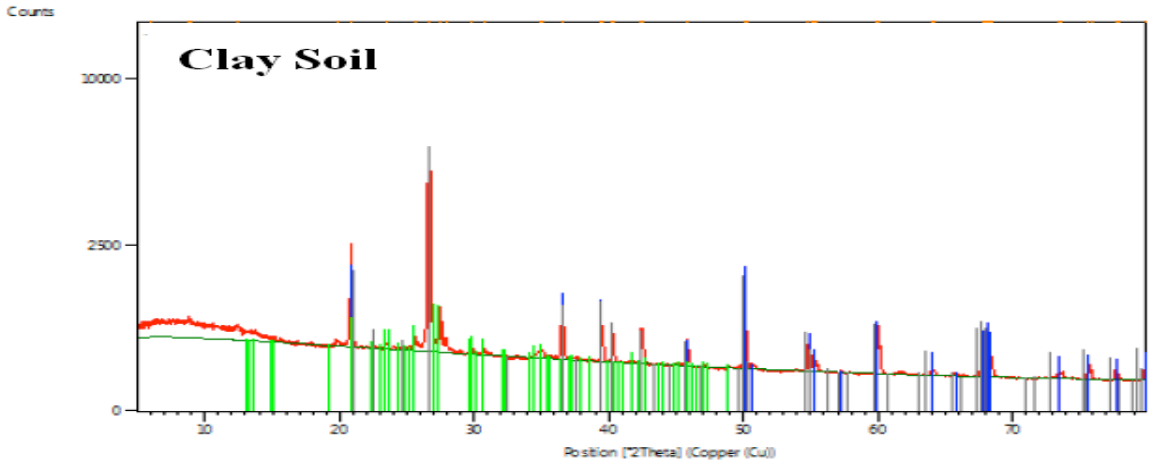

Figure 4.5. XRD spectroscopy of the model silt-clay soil particle size < 0.063mm

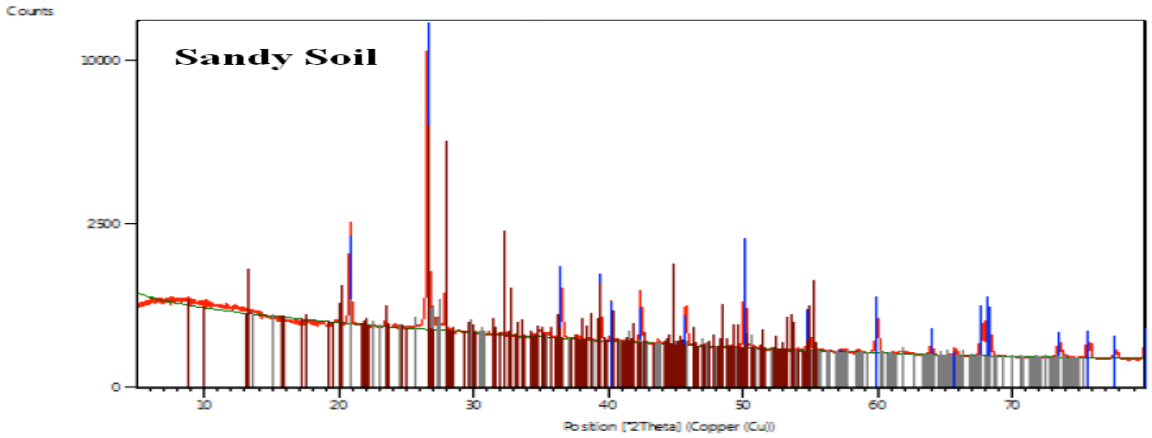

Figure 4.6 XRD spectroscopy of the model sandy soil particle size (0.25-1.00 mm).

Table 3.2 shows the mineralogical composition of the different particle sizes from the x-ray Diffraction (XRD) analysis.

Soil type	Particle sizes (mm)	Record mean Score	Formula	Compound name
Silt-clay	< 0.063	67 16 42	SiO_2 $KAlSi_3O_8$ $AlPO_4$	Quartz -low Feldspar Aluminium Phosphate
Sandy	0.25-1.00	77 12 15	SiO_2 $KAlSi_3O_8$ $CaCuO_7$	Quartz –high Orthoclase Calcium Copper Oxygen

4.3.4. Scanning electronic microscopy (SEM) analysis of the soil

SEM analyses of the silty-clay and sandy soil samples were conducted to present the microscopic structure of their particle distribution (**Figure 4.7**). The silt-clay soil particle showed a smoother nature of the particles as shown in **Figure 4.7.A** while the sandy soil particle had a coarse surface structure as shown in **Figure 4.7. B.** The structural representation of the different particles will help broaden the understanding on how bacterial attach to the soil during the soil bioremediation processes.

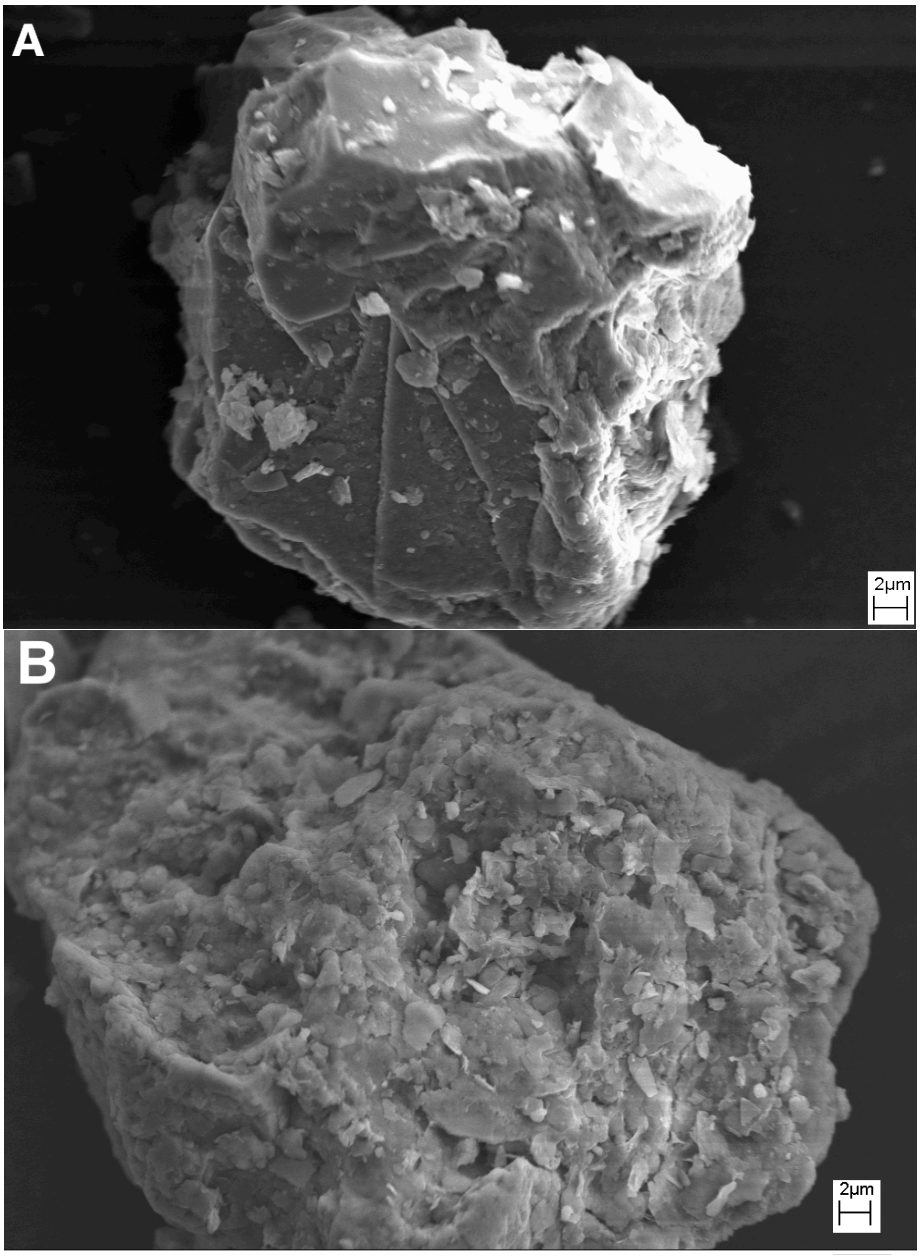

Figure 4.7 SEM of the Niger Delta modelled silt-clay (A) and sandy (B) soil

4.4 DISCUSSION

The characterized and separated Hilton soils based on the particles size were used to model the clay and sandy soil of Niger Delta. These two soils were chosen based on the investigation of six different representative soil profiles that established that clay and sandy soil are the most predominant soils types in the region (Akpokedje, 1987). The choice of soil from the Niger Delta region is as a result of the challenges of oil pollution in the region (Sojinu *et al.*, 2010).

The physiochemical analyses of the different graded soil (**Appendix II-Table II-1b**) and the modelled clay and sandy soil (Table 3.1) showed a slightly acidic soils types. The pH result of the clay and sandy was 6.73 ± 0.14 and 6.45 ± 0.13 respectively. Fullen *et al.,* (2011) reported a slightly more acidic pH 5.35 of the Hilton soil while investigating the effect of soil properties. Iwegbue *et al.,* (2006) while assessing the soils of an oil field in the Niger Delta reported that the soil pH during the wet season range between 3.51-6.83 and during the dry season 4.29-7.32. The Niger Delta soils can be averagely described as slightly acidic which is similar to that of the Hilton soil based on the findings.

The organic carbon observed in the clay soil was 26.4 ± 0.3%, which was higher than the 19.4 ± 0.2% observed of the sandy soil. Fullen *et al,* (2006) reported of a strong correlation between soil organic matter and cumulative particle fraction of both clay and silt soil from the Hilton. Periodic sampling of the topsoil of the Hilton soil suggested that clay content and increased the coarse fraction (Fullen and Brandsma, 1995). This could have been responsible for the higher organic carbon observed the clay soil from the Hilton. The nutrient level in terms of the % nitrogen and phosphate in the modelled clay and sandy soil from the physiochemical result was slightly low to enhance bioremediation processes (Table 3.1). Nutrient like nitrogen and phosphorus are very important for bacterial proliferation during the bioremediation of oil (Ayotamuno *et al.* 2006).

The Niger Delta region is underlined by different superficial soil deposit ranging from the organic mostly peaty marine mud or chicoco in the tidal flat or saline mangrove swamp to the active silty clay of the freshwater back swamps deltaic lateritic soils of the dry flatland or plains (Leton and Omotosho, 2004). The soil texture in region varied widely from sandy to predominantly silty loam, and sandy clay loam with a mixed clayey/silty loam texture Effiong and Ayolagha (2010) and are mainly silty clay and were relatively acidic (Ayotamuno *et al.* 2006).

The major elements identified in the Hilton soil are sodium, magnesium, aluminium, potassium, iron and silicon see **Figure 4.3**. Other trace elements such as (Cr, Ni, Ba, Nb, Zr, Y, Sr, Rb, Co etc) were also identified as shown in **Appendix II-Table II.2**. The XRD analyses of the modelled clay and sandy soil types revealed that the predominant minerals as quartz - SiO_2 with some percentages of kaolinite (Orthoclase), illite and smectite (Feldspar) (**Table 3.2).** Feldspar is a group of smectite identified. Smectite is a commonly occurring expanding lattice mineral that shrinks and swells during drying (Phillips *et al*., 2011). Soil permeability and pore and fractures in the rock are critical factors controlling the movement of water from the surface into the underlying layers (Atlas *et al.,* 2005). Olorunfemi, (1984) reported such similar mineralogical composition of the Niger Delta soil. He observed that the major minerals found in the region are quartz and kaolinite with some proportions of smectite, goethite and gibbsite **(Appendix II-Table II.1).** The central delta is essentially quartz – kaolinite zone while the eastern delta has the highest concentration of highly aluminous smectite. The soil type plays an important role in the fate of petroleum crude oil contaminant (Stemple *et al*., 2003). Hydrocarbon breakdown is influenced by the soil environment and soil fractions, such as the surface area, composition of surface fractions and reactivity (Scherr *et al.,* 2007).

The modelled soils were maintained within the temperature of 25-30 ^{o}C in the sterile bags to model the temperature of the Niger Delta environment. The ambient environment in the Niger Delta has a daily mean minimum and maximum temperature of 23 ^{o}C and 31.5 ^{o}C respectively (Ayotamuna *et al.*, 2006). Maintaining the soil within the temperature range 25-30 ^{o}C was crucial for the soil bioremediation phase of this investigation.

4.5 INTERIM CONCLUSIONS:

It is concluded that:

- Geochemical properties of the soil sample from the XRF showed major elements are sodium, magnesium, aluminum, potassium, and iron with silicon having high percentage.
- XRD analysis revealed minerals such as quartz, kaolinite (Orthoclase), illite and smectite (Feldspar), which are similar to those of the Niger Delta.
- The modelled silt-clay and sandy soil are similar to that of the Niger Delta based on the physicochemical and particle size characteristic (Akpokedje, 1987), geochemical properties (Olorunfemi, 1984) and environmental temperature (Ayotamuna *et al.*, 2006).

CHAPTER 5

BIOREMEDATION OF MODELLED PETROLEUM OIL-CONTAMINATED SOILS OF THE NIGER DELTA

5.1 INTRODUCTION:

The soil used for this investigation was that which had been characterized and separated based on the particles size and then restructured to model that of Niger Delta soil **(Chapter 4).** The choice of soil from the Niger Delta region was as a result of the challenges of oil pollution in the region and to provide an understanding of the influence of the bacterial consortium (*Pseudomonas sp and Rhodococcus sp* characterized in **Chapter 3)** and zeolite on the oil removal from the soil (preliminary investigation).

Further investigation on the effects of soils structures on bioremediation was carried out using two-modelled Niger Delta soil particle size composition (silt-clay and sandy). These two soils were chosen based on the investigation of six different representative soil profiles that established that clay and sandy soil are the most predominant soils types in the region (Akpokedje, 1987). The bacterial consortium used for this investigation was composed of: *Pseudomonas aeruginosa, Rhodococcus sp, and Acinetobacter sp*. (*Acinetobacter sp* was included to this investigation to enhance the bioremediation process due to its utilization in previous studies).

Natural zeolite and its modified forms have been used to remove organic contaminants from the environment (Wang and Peng, 2010; Gunter and Zanetti, 2000). The zeolite (clinoptilolite) has been reported as a source of slowly release of potassium and nitrogen to the environment when needed and also act as water moderator, in which they can absorb up to 55% of the weight in water and slowly release it under the demand of plant and the ecosystem (Mumpton, 1999). Little to no information has been reported with regards the combined influence of bacteria and zeolite in remediating the soil environment of contaminated crude

oil which this research will be investigating. The zeolite used for this study was identified as clinoptilolite from XRD analysis. An understanding of the extent and nature of the influence of these two-modelled soils particle size soil composition and zeolite augmentation on degradability of petroleum hydrocarbon mixture in soil mixture will greatly help in developing a remediation strategy in the Niger Delta region.

5.1.1 Hypothesis of this investigation

The following hypotheses were considered during the investigation:

1. Does the bacterial consortium effectively bioremediate petroleum contaminated soil?
2. Does soil particle size composition have any influence on the level of bioremediation of a crude oil-contaminated soil?
3. What influence does zeolite have on the level of bacterial growth and its bioremediation potential in a crude oil contaminated soil?

5.2 METHODS:

5.2.1 Preparation of standard inoculum and microbial consortium

The method of Ghazali *et al*., 2004 was adopted with some modification. Individual cultures of the characterized bacteria were separately grown on 1% v/v crude oil- containing BSM in a shake flask for 5-6 days and subsequently transferred into another 1% v/v of crude oil mineral salt solution for 18 h at 30°C in an orbital shaker at 150rpm prior to inoculation. The cells were harvested by centrifugation, rinsed three times with sterile saline before being re-suspended in 5mls sterile BSM to yield an absorbance reading of 0.5 at 540nm and cell count was determined. 10% (v/w) of the resulting colony forming unit (CFU)/g soil was used as a soil inoculum (with the cell count between 1.9×10^7 and 2.9×10^7 cfu/g). The microbial consortium was formulated by mixing equal proportions of pure bacterial cultures (resulting to a final mixed culture inoculum cell population of 2.5×10^7 cfu/g).

5.2.2 Determination of microbial population from the crude oil-contaminated soil

1-gram soil samples were aseptically taken from the soil treatments in the flask (after thoroughly mixing the soil sample using ethanol sterilized spatula) and transferred into 9 ml of Ringer solution. The soil solution was thoroughly shaken on a vortex mixer for 2-3 min and then the soil particulates were allowed to settle for a minute. Serial dilutions from the resultant suspension of 10^{-1} to 10^{-8} were performed by dispensing 0.5 ml of sample in 4.5 ml of sterile $1/4^{th}$ strength ringer solution. 20 µl of each dilution was dispensed onto TSA plates under aseptic conditions using the Miles and Misra technique in triplicate. The TSA plates were then incubated at 30°C for 24hrs. Samples for bacterial viable counts were taken at 0, 7, 14, 21 and 30 days and the number of colony forming units counted.

CFU/ml was calculated with the formula: CFU/ml = n x (1/ (sample volume (ml))) x 1/D.F
Where n is the no. of colonies and D.F. is dilution factor.

5.2.3 Extraction of residual oil from the crude oil-contaminated soil

The entire oil-contaminated soil flask contents (less of the 1 gram used for the microbial analysis) were carefully transferred into the thimbles and place in the extraction chamber, which is suspended above the flask containing the solvent-hexane. Additional 10mls of hexane was introduces into the flask and thoroughly shaken and added into the extraction chamber and the extraction processes was carried-out by soxhlet extraction. The extraction was allowed to run for a period of 5 h. The extract was subsequently evaporated to approximately 1 ml using a rotary evaporator –Rotavapor RE150 at 35 ^{o}C **(see Figure 5.1)**; this was to further remove any trace of hexane in order to weigh the petroleum crude oil.

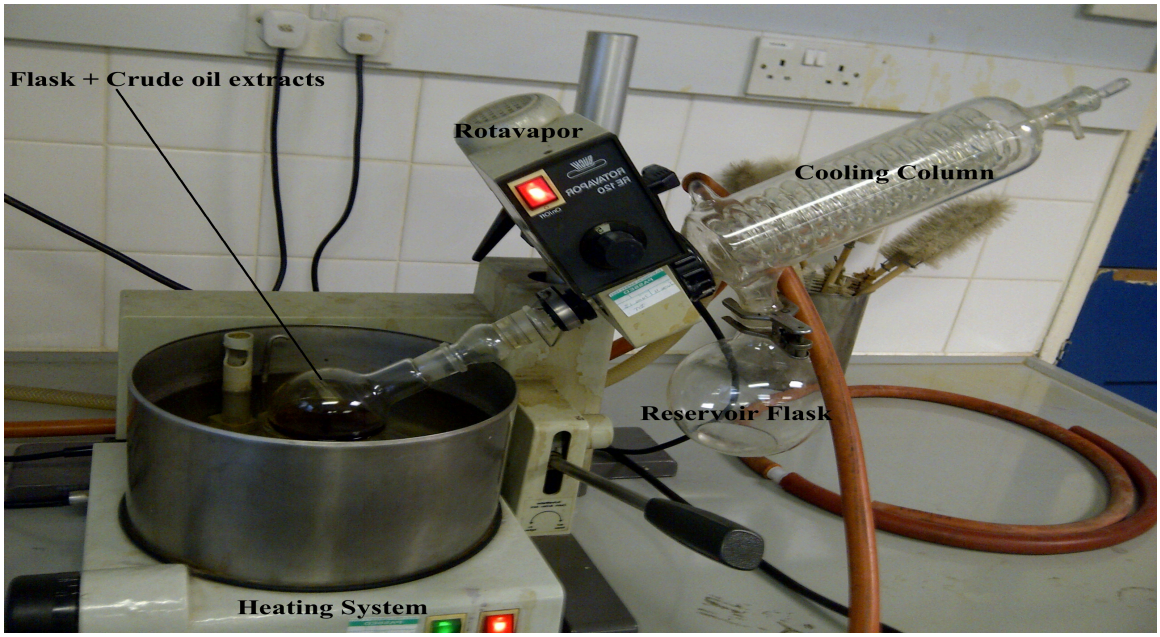

Figure 5.1. A rotary evaporator used for the evaporation of hexane from the crude oil extracted from the soil

5.2.4 Quantitative analysis hydrocarbon- Gas chromatography

The determination of hydrocarbon in the soil was performed on the extracted residual oil samples and standards using the Thermo Finnigan, Trace GC Ultra fitted with a flame ionization detector and capillary column type Rtx-5MS (30x0.25mm); run on auto-sampling method and alkanes were detected using a standard solvent of known hydrocarbon. The initial oven temperature was held at 50°C for 2minutes while the injector and detector temperature were maintained at 270°C. The oven was programmed to rise from 50°C to 270°C at 10°C/min increment and held at 270°C for 15 mins. The injector was run on Splitless Splitless mode while 1 µl of the sample taken from the vial bottom was analyzed. Hydrogen flow was maintained at 35 ml/min, airflow at 350 ml/min, and makeup gas nitrogen at 30 ml/min while the carrier gas used was helium. The quantification of hydrocarbon was determined by comparing the peak area of the individual hydrocarbons to that of the standard alkane.

5.2.4 Recovery of oil and quality control

Petroleum hydrocarbon extraction was carried out using the soxhlet extraction technique. To ensure good quality control of the extraction technique, clean soil samples were spiked with

replicate known amount (0.05 – 1.00 gram) of crude oil in replicate and subsequently extracted. The extraction results for 0.05 – 1.00 g petroleum crude oil show a linear relationship **(Figure 5.2)** with good reproductively and an extraction efficiency of 95%.

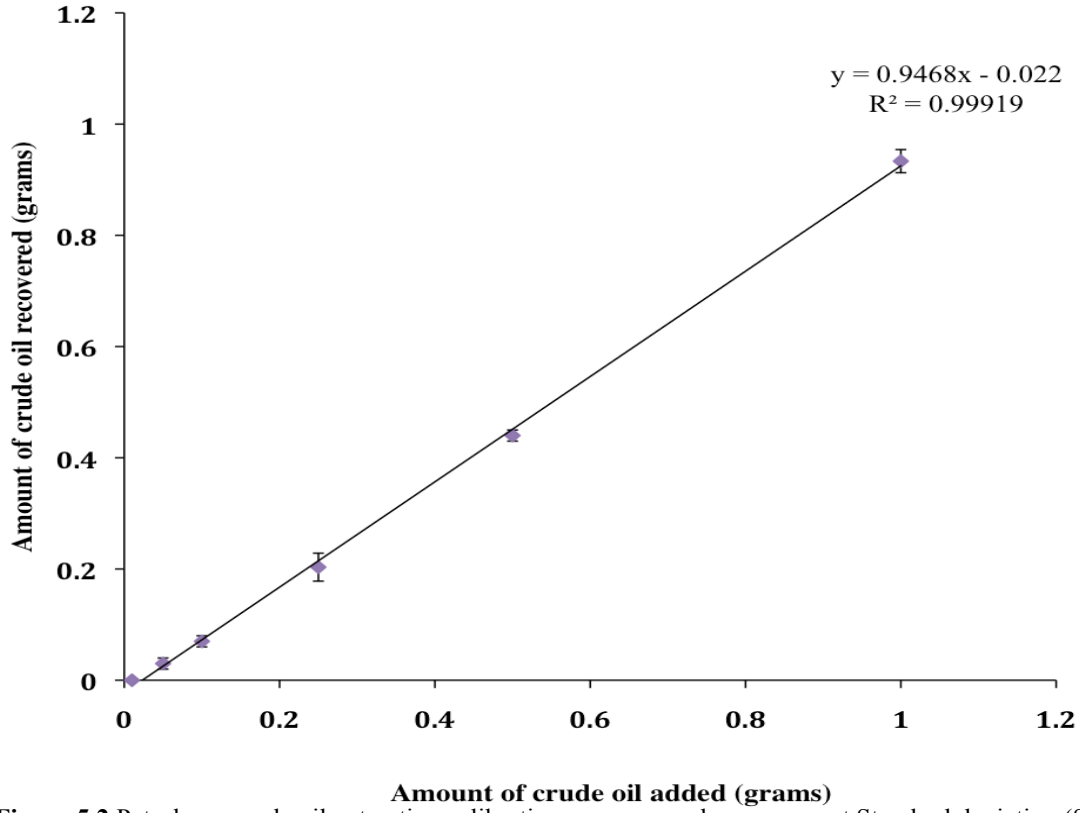

Figure 5.2.Petroleum crude oil extraction calibration curve- error bars represent Standard deviation (SD) (n=3)

5.2.5 Bioremediation of crude oil-contaminated soil using bacteria and zeolites (Preliminary investigation).

The biodegradation experiments were conducted using replicate 100 ml flask containing 30 grams of sterile soil (not amended with zeolite) and another same flask with 0.05% w/w zeolite added, both autoclaved at 121°C for 20 mins. The sterile soil samples were spiked with 0.5 ml of petroleum crude oil. 5mls of sterile distilled water and 0.5ml basal salt medium were aseptically introduced into each of the flask. A mixed culture of bacterial consortium (*Pseudomonas sp* and *Rhodococcus sp*) previously characterized in chapter 3 was used for this investigation. 10% (v/w) resulting colony forming unit (CFU)/g soil of the bacterial consortium was used ($2.5 \times 10^7 \pm 0.4$) cfu/g (as determined in section 5.2.1). The experiments were conducted with the following treatment combinations in replicate: oil-contaminated soil

amended with zeolite and without zeolite both with bacterial consortium; control experiments: soil with bacterial consortium; oil-contaminated soil and soil (un-contaminated with oil)

The experiment was carried out for a period of 30 days in an incubated water bath at 30 ^{o} C. Water losses due to evaporation were compensated at two days interval by addition of sterile distilled water on weight bases. Two hours after the soil had been amended with crude oil and bacterial consortium, samples were taken for initial microbial cell population and the crude oil concentration determined (as described in section 5.2.3). Further samples were carried out at day 7, 14, 21 and 30.

The microbial population was determined using the Miles and Misra technique in triplicate (as described in Section 5.2.2) and the oil was extracted from the entire oil-contaminated soil flask contents (less of the 1 gram used for the microbial analysis) by soxhlet extraction with 150 ml hexane for a period of 5 h. The extract was subsequently evaporated to approximately 1 ml using a rotary evaporator –Rotavapor RE150 at 35 ^{o}C. The quantitative analyses of the extracted residual oil samples was performed using the Thermo Finnigan, Trace GC Ultra fitted with a flame ionization detector and capillary column type Rtx-5MS (30x0.25mm).

5.2.6: Influence of soil particle size composition on bioremediation of crude oil-contaminated soils.

The soil experiments were conducted with the following treatment combinations in replicates using clay and sandy soil separately **(See Table 5.1)**

Table 5.1 Soil treatments experimental combinations for the remediation process

Soils treatments of the individual soil types (Clay and Sandy)	Represents
20 grams sterile soil + 0.5 ml crude oil + 2 ml bacterial consortium + 2 grams sterile zeolite + 5ml BSM	S+C+B+Z
20 grams sterile soil + 0.5 ml crude oil + 2ml bacterial consortium + 5 ml BSM	S+C+B
20 grams sterile soil + 0.5 ml crude oil + 7 ml BSM	S+C (Control)
20 grams sterile soil + 0.5 ml crude oil + 2 grams sterile zeolite + 7 ml BSM	S+C+Z (Control)
20 grams sterile soil + 7 ml BSM	S (Control)

The biodegradation experiments were conducted in the laboratory controlled environment (same as described in Section 5.2.2) except with some slight modification. Replicate 100 ml flask containing 20 grams of the individual sterile soil (clay & sandy) composition (autoclaved at 121°C for 20 mins) treatments (see Table 5.1) were conducted under the same condition for a period of 30 days in the incubation water bath at a temperature of 30 ^{o}C. 10% standard inoculum (2.52 x 10^{7} cfu/g soil) of three bacterial consortium (*Pseudomonas aeruginosa, Rhodococcus sp, and Acinetobacter sp*) was utilized during the investigation (*Acinetobacter sp* was added to the preparation as a result of further characterization in **Chapter 3** and to enhance the bioremediation process). Sterile BSM was added at intervals of three days on weight bases to compensate for moisture loss (which was determined by weighing each of the flask content) and to maintain nutrient replacement. Two hours after the soil had been amended with crude oil and bacterial consortium, samples were taken for initial microbial and concentration determination. Further samples were carried out at day 7, 14, 21 and 30 using the Miles and Misra technique in triplicate **(see section 5.2.2)** for the microbial analyses while the residual oil from the entire oil-contaminated soil flask contents (less of the 1 gram used for the microbial analysis) was extracted with hexane using the Soxhlet extraction for 5 hr **(as described in 5.2.3).** The quantitative analysis of the extracted oil were carried out by weight measurements **(as described 5.2.1 iii)** .The determination of hydrocarbon in the soil was performed on the extracted residual oil samples and standards using the Thermo Finnigan, Trace GC Ultra fitted with a flame ionization detector and capillary column type Rtx-5MS (30x0.25mm) **(as described 5.2.1 vi)**. The GC was run on auto-sampling method and alkanes were detected by using a standard solvent. The quantification of hydrocarbon was determined by comparing the peak area of the individual hydrocarbons to that of the standard alkane.

5.2.7 Statistical analyses

All the experiments were performed in replicate and the means and standard error of the mean was determined using graph pad prism. Experimental data were analysed using column analyses: "t-test: sample (comparison of means), one-way ANOVA and using grouped analyses: Two-way ANOVA, row means with standard deviation, and multiple t tests. All significant difference (p) values were determined and data interpreted.

5.3 RESULTS ON BIOREMEDATION OF CRUDE OIL-CONTAMINATED SOIL (PRELIMINARY INVESTIGATION)

A preliminary investigation of bioremediation of crude oil-contaminated soil using bacteria and zeolite was conducted. The bacterial consortium used for this investigation comprised of *Pseudomonas sp and Rhodococcus sp* characterized in **Chapter 3.** This investigation was carried-out in order to provide an understanding of the impact of bioremediation on the bacterial consortium cell population and the oil removal rate from a modelled Niger Delta soil formulated in **Chapter 4**.

5.3.1. Microbial consortium cell population:

The soil sample contaminated with the crude oil petroleum hydrocarbon had an initial bacterial consortium population of 2.5 x 10^7 cfu/g **(Figure 5.3).** The control soil sample was amended with the addition of only the bacterium consortium without the crude oil as the carbon source. The bacterial population in all the samples showed no significant changes in population size during the first 14 days. Although, minor differences were observed between samples that were within normal variation and hence not significant since there were no apparent growth of bacteria in the time period. It was concluded that this might have been due to lack of nutrients. Consequently, 2 ml BSM was used to replace the sterile distilled water at day 14 of the various experimental treatments. Within a further 7 days (Day 21) the viable

counts slightly increased to 5.6 x 10^7 cfu/g and 4.5 x 10^7 cfu/g for soil treatment with zeolite and without zeolite respectively. At the 30th day of investigation there was an increase in the cell population to 9.98 x 10^7 cfu/g and 9.2 x 10^7 cfu/g in oil-contaminated soil treated with zeolite and without zeolite respectively while the control sample without petroleum crude oil was to 1.7 x 10^7 cfu/g indicating no growth.

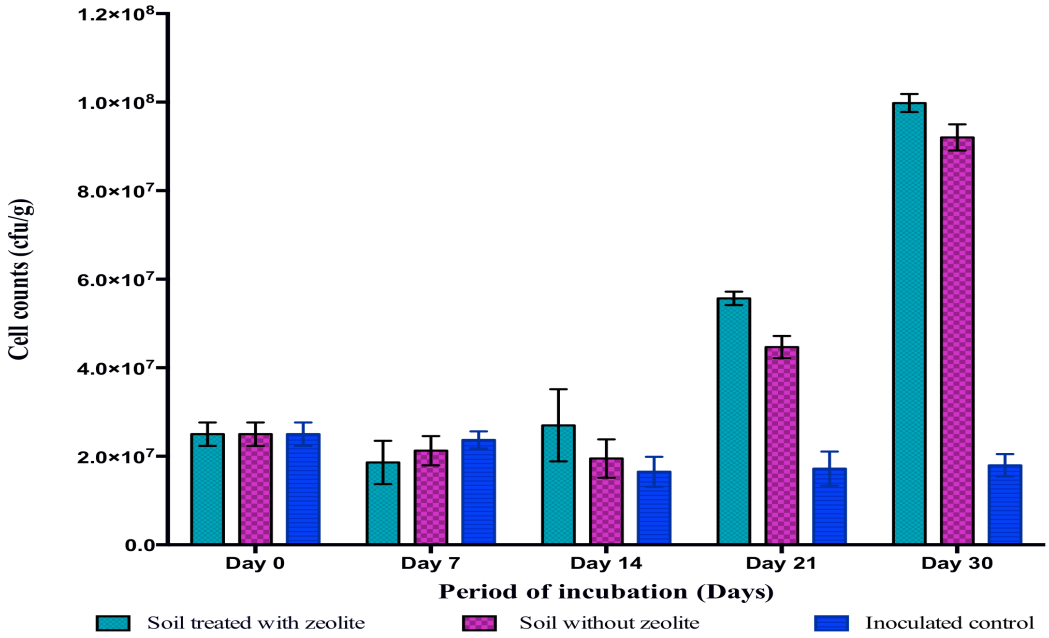

Figure 5.3 Growth patterns in colony-forming units of bacterial consortium in oil contaminated soil treated with zeolite and without zeolite. Error bar represents SD of replicated experiments (n=3)

5.3.2. Hydrocarbon removal

The quantification and oil removal patterns of the petroleum hydrocarbon in the soil were based on the comparisons of the hydrocarbon compositions extracted from the soil at the beginning of the investigation (day 0) and at day 30. These comparisons were analysed based on the weight analysis of the residual oil and by gas chromatographic analysis with the reference standards.

5.3.2.3. Quantification of residual oil: Weight analysis methods

Residual oil was quantified by weighing the extracted oil obtained by soxhlet extraction. Soil amendment experiment showed a more rapid and greater extent apparent oil removal with the addition of both bacterial consortium and zeolite. At the beginning of the study (day 0), 97.8

± 0.4% and 98.2 ± 0.9% of oil-contaminated soil treated with zeolite and without zeolite respectively were recovered **(figure 5.4).** After 7 day of incubation with the bacterial consortium, 61.7% ± 0.71% and 77.7% ± 1.90% of oil-contaminated soil treated with zeolite and without zeolite respectively were recovered. In contrast, control experiments without bacteria showed only a slight reduction down to 90.9% ± 2.1% and 92.4% ± 0.7% for soil treatment with and without zeolite. This suggests that the zeolite would have adsorbed some of the crude oil contaminates and that bacterium consortium would have metabolizing the crude oil without growing. The reduction in oil continued such that by day 30, 79% of oil had been removed in soil treated with zeolite and bacteria, and bacteria in soil without zeolite had removed 67% of oil.

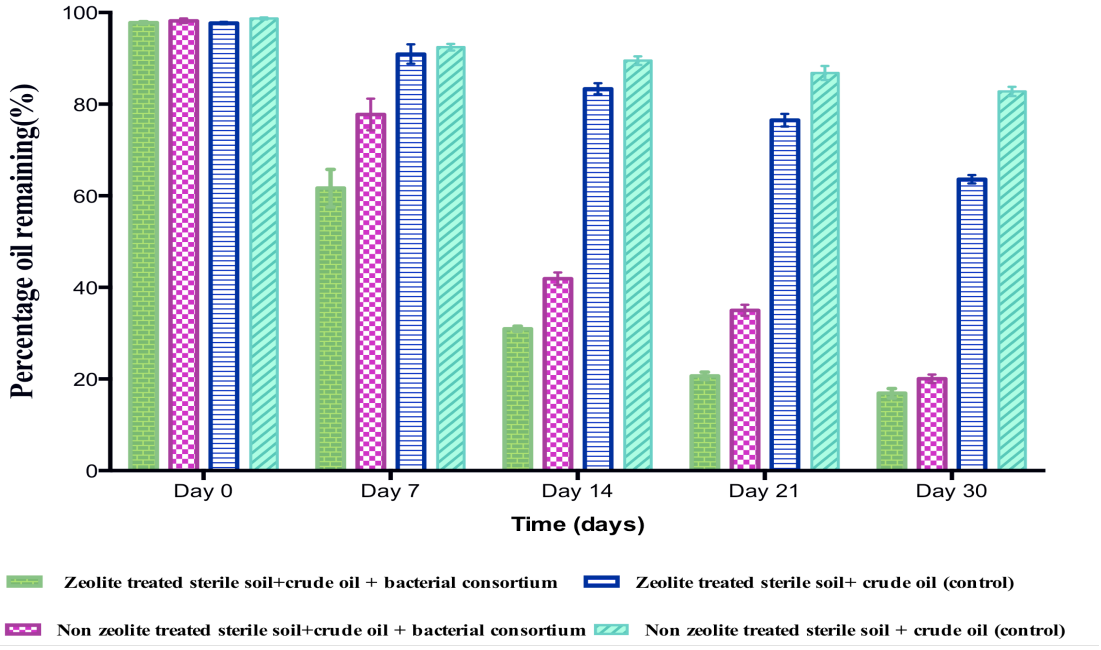

Figure 5.4 Shows the impact of addition of zeolite and bacterial consortium (*Pseudomonas sp and Rhodococcus sp)* upon oil removal rate at 30 °C for a period of 30 days. Error bar represents SD of replicated experiments (n=3)

The control experiments (without bacterial consortium) with zeolite and without zeolite by day 30 of the investigation showed 34% and 16% oil removal respectively. This removal could be attributed to abiotic factors either by volatilization or during incubation in the experimental set-up in the incubator at 30°C, or adsorption onto the particulate material during the extraction process using hexane and loss to the environment during analysis

evaporation. The statistical analyses **(Appendix III-Table III.1)** show that the additions of bacterial consortium significantly ($p < 0.0001$) enhanced oil removal in the presence or absence of zeolite; that addition of zeolite to soil significantly enhanced oil removal in the presence of bacteria ($p < 0.0001$).

A summary description of the influence of zeolite on the bacterial consortium **(as described in figure 5.3)** and oil removal pattern **(Figure 5.4)** from the preliminary investigation were further combined and described in the graphical representation **(Figure 5.5)** to present a better understanding of the bioremediation process.

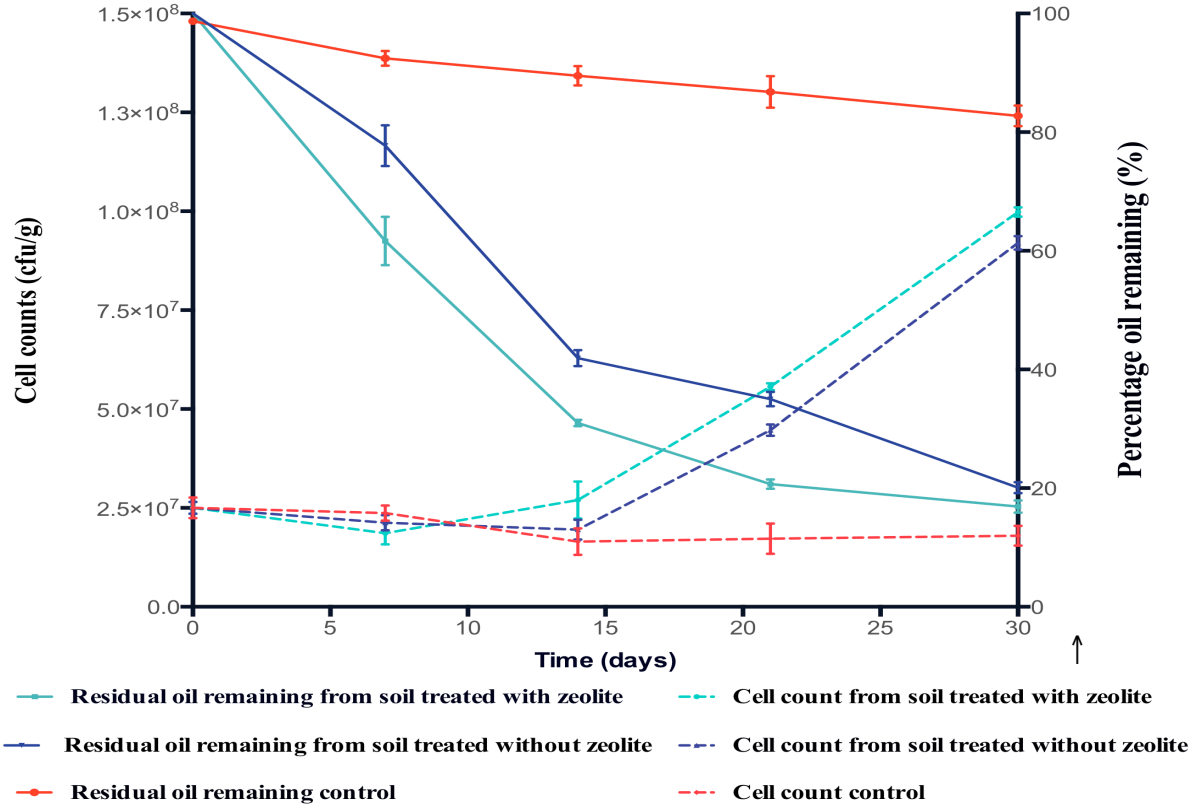

Figure 5.5 Bacterial growth and oil removal pattern in a soil contaminated with 1.5% w/v crude oil soil with addition of zeolite incubated at 30 °C for a period of 30 days. Error bar represents SD of replicated experiments (n=3)

5.3.2.4. Qualification of residual oil: Gas chromatographic analysis

A gas chromatographic analysis of the hydrocarbon extracted with hexane at the start of the investigation identified aliphatic compounds with carbon numbers between C_{12}-C_{28}. The chromatograms of degraded oil recovered from the various soil treatments at the start of the investigation (time=o) and the end day 30 and controls are presented in **Figure 5.6**. The

reduction in the peak area and heights from the chromatograms from the various extracts is an indication of reduction in the concentrations of the hydrocarbon components in the extracts.

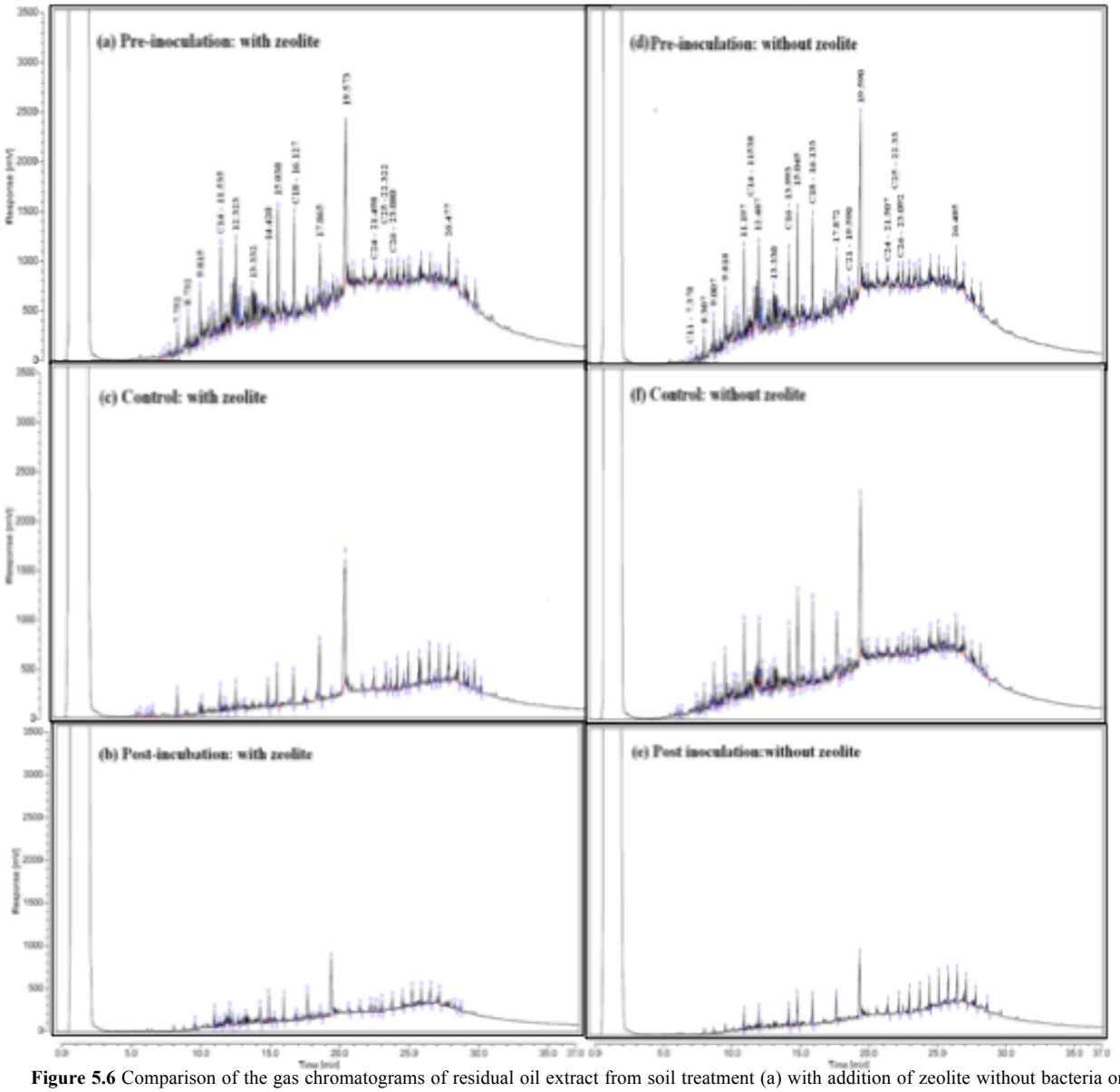

Figure 5.6 Comparison of the gas chromatograms of residual oil extract from soil treatment (a) with addition of zeolite without bacteria on day 0. (b) With addition of zeolite and bacterial consortium on day 30. (c) With addition of zeolite without bacterial consortium on day 30 (d) without bacteria on day 0. (e) With bacterial consortium on day 30. (f) Without bacterial consortium on day 30.

The peak areas from the selected hydrocarbon peaks were evaluated in relation to % removal of individual hydrocarbons. Quantitative analysis of the residual oil extracted from the soil at day 30 **(Figure 5.7)** of the investigation revealed that C_{12} and C_{16} hydrocarbon were degraded the most. C_{12} was 97% degraded in soil treated with zeolite whereas 78% of the hydrocarbon component C_{12} was degraded in soil not treated with zeolite. C_{16} showed mean percentage

removal of 92% and 88% for soil treated with and without zeolite respectively. Surprisingly, C_{14} hydrocarbons were only degraded by 42% and 56% in the presence of bacteria in soil without zeolite and zeolite treated soil respectively. C_{18} - C_{25} hydrocarbons were partly removed in both soil treatments. Hydrocarbon component (C_{26} and C_{28}) were partially degraded by the bacterial consortium in the case of extracts from soil not treated with zeolite to mean percentage of 33% and 27% respectively while soil treated with zeolite and bacterial consortium showed mean percentage removal of 36% and 29% respectively. Control experiments of oil-contaminated soil without bacteria and zeolite amendments show partial removal of the lower hydrocarbon with C_{12} - C_{16}.

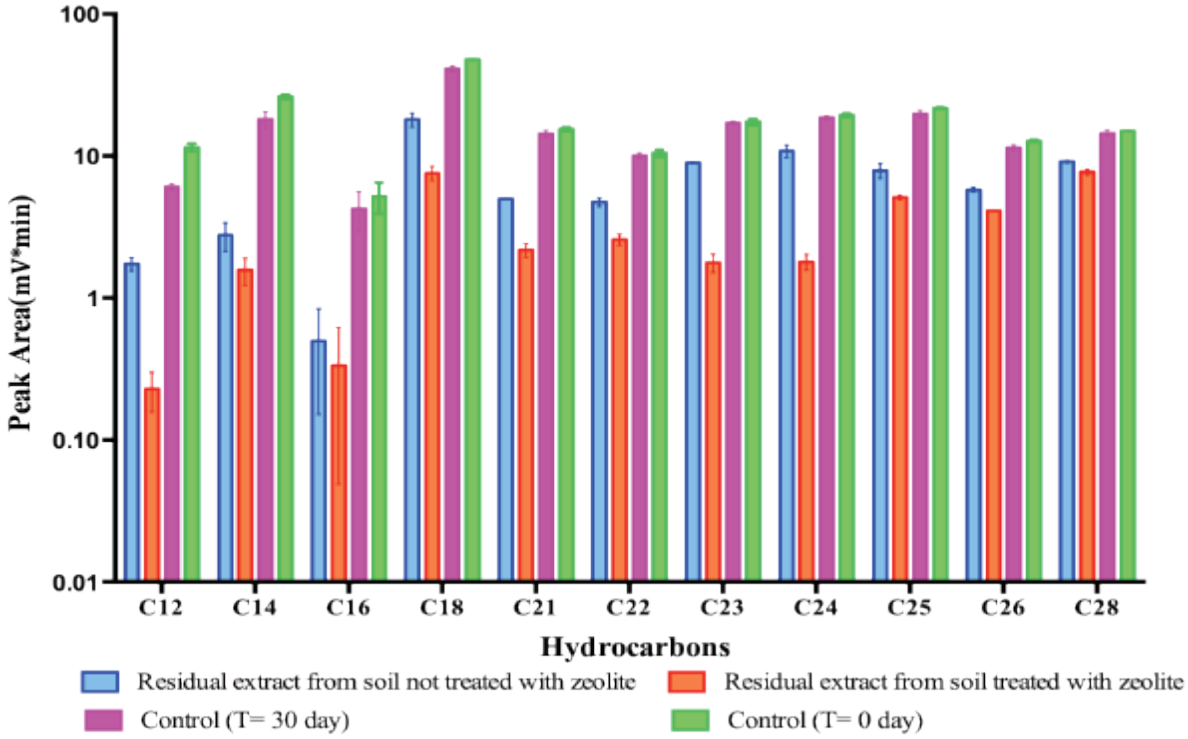

Figure 5.7 A column chart showing peak areas of hydrocarbons found in GC analyses of residual crude oil extract from soil not treated with zeolite but with bacteria, soil treated with zeolite and bacteria and soil not treated with zeolite and bacteria (control) at day 0 and day 30. Error bar represents SD of replicated experiments (n=3)

5.3 RESULTS ON THE INFLUENCE OF SOIL STRUCTURE ON BIOREMEDATION

These results were obtained from the investigation conducted to establish the influence of soil particle sizes (clay and sandy) on crude oil bioremediation using bacterial consortium and zeolite. Theses soils were characterized (**see Table 5.2**) and reformulated modelling the Niger Delta soils. The bacterial consortium used for this investigation was composed of a

mixture *Pseudomonas aeruginosa, Rhodococcus sp, and Acinetobacter sp*. *Acinetobacter sp* was added to this experiment to enhance the bioremediation process. The zeolite used for the investigation is characterized in **Table 5.3**.

Table 5.2 Profile description and characterization of the soils used for the investigation

Physical appearance	**Physiochemical properties**	
Particle name	Silty clay	Fine sandy
Particle sizes (mm)	< 0.063	0.25-1.00
pH	6.73 ± 0.14	6.45 ± 0.13
% Moisture by mass (% w/w)	25.4 ± 0.2	22.6 ± 0.20
Available PO_4 (ppm)	1.60 ± 0.12	1.30 ± 0.03
Nitrogen (% w/w)	0.17 ± 0.01	0.14 ± 0.04
Organic Carbon (% w/w)	26.4 ± 0.3	19.4 ± 0.40

Results represent the mean±standard deviation of three replicates (n=3)

A summary of the physiochemical properties of the zeolite analyses is described in Table 5.3.

Table 5.3 Profile description and characterization of the zeolite used for the investigation

Physical appearance/ Element	**Physiochemical properties**
Zeolite name	Clinoptilolite
Particle sizes (mm)	1.00-2.00
Nature of substance	Granular pallets
pH	6.20
Phosphorus (%)	<0.00072
Silicon (%)	5.906
Potassium (%)	0.984
Trace elements (%)	Present

Soil type is an important factor to consider when determining the best-suited bioremediation approach to a particular situation. Soil augmentation with zeolite in the two different soil types exhibited two dissimilar growth patterns in terms of the bacteria cell population **(see figure 5.8 and 5.9)**.

5.4.2 Influence of zeolite (clinoptilolite) and bacteria on the oil removal pattern in silt-clay soils

The clay soil sample with and without addition of zeolite contaminated with the crude oil petroleum hydrocarbon had an initial bacterial mean population of 2.39 x 10^7 cfu/g and 2.44 x

10^7 cfu/g respectively **(Figure 5.8).** The bacterial population in the both clay soil treatments showed a slight increase but not a significant difference in population size between both soils during the first 7 days. However, there was a significant difference ($p < 0.0001$) in the oil removal between both soil treatments during this period. The clay soil with the zeolite had 69.0% ± 0.3% oil remaining while that without zeolite had 84.8% ± 1.8% remaining respectively. The p-value (< 0.0001) also showed a significant difference between the two treatments **(Appendix III-Table III.2)**. Interestingly, the control experiment also showed a slight but not significant increase ($p > 0.0001$) in both bacterial cell population sizes (at day 7 sampling) and oil removal. The bacterial cell population for the control after then began to gradually decrease during the period of the investigation. At day 14, test flasks maintained an increase in bacterial cell population and decrease in oil level in both clay soil amendments, with significant difference between the clay soils with and without zeolite (46.4% ± 1.5% and 67.2% ± 1.0%, respectively). This significant oil removal and increase in bacterial population was maintained on day 21 of the investigation. At day 30, the clay soil with and without zeolite but with addition of bacterial consortium showed a significant difference in the oil removal, they had 10.6% ± 1.4% and 23.8% ± 1.0% oil remaining respectively. Statistical analysis of the results at day 30 **(Appendix III-Table III.3)** confirmed that there was a significant difference ($p < 0.0001$) in oil removal caused by zeolite in the presence and absence of bacteria. Also at day 30, clay soil spiked with crude oil amendments (control) with addition of zeolite but without bacterial consortium had oil remaining of 67.2% ±0.6% while the crude oil spiked clay soil without both treatments had 89.1% ± 2.0% oil remaining.

A clear pattern of oil removal as time progressed with and without zeolite and bacterial addition was observed in the clay soil. The rate of oil removal in the absence of both zeolite and bacterial was minimal over the 30 days period.

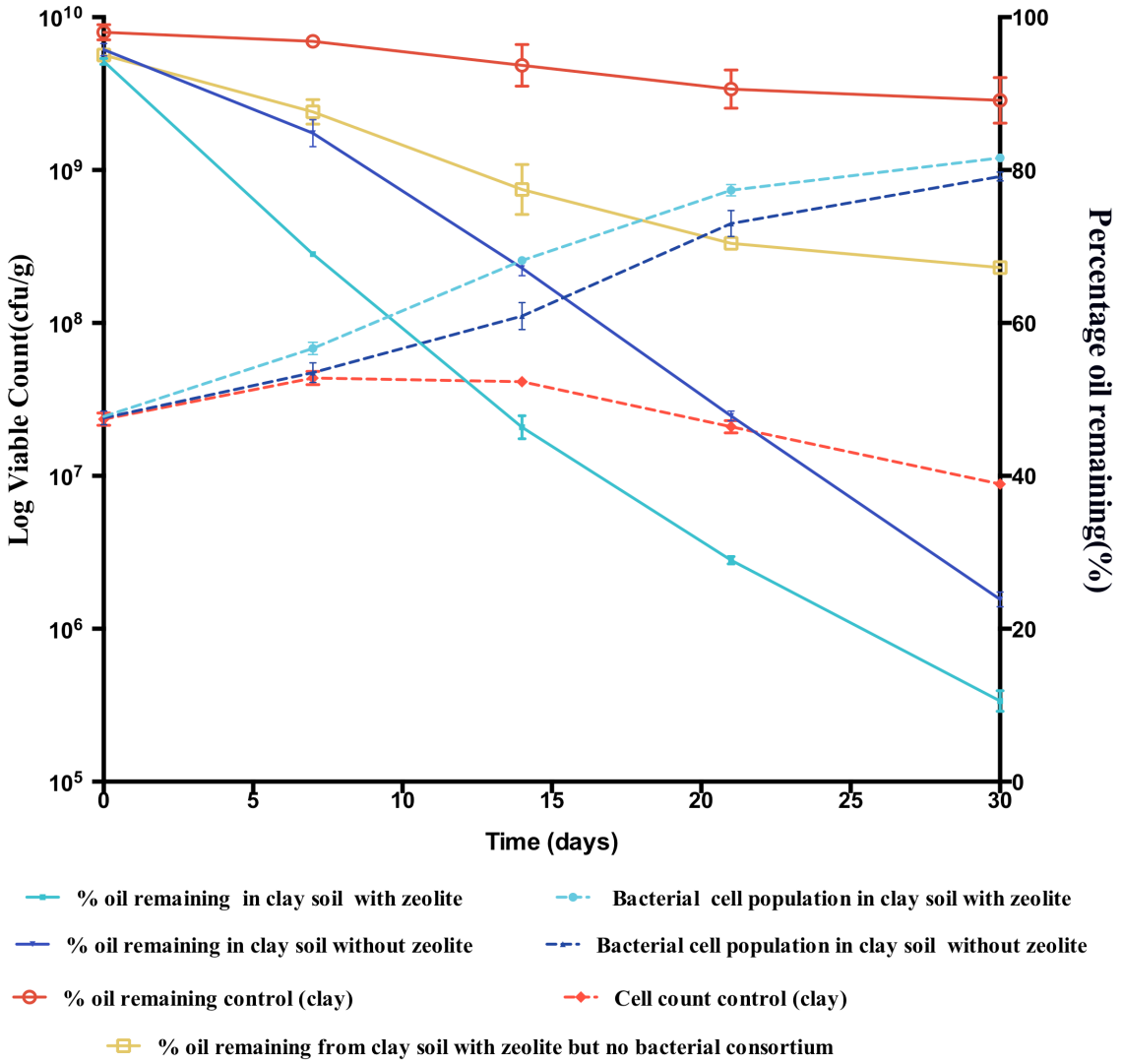

Figure 5.8 Bacterial growth and oil removal pattern in a clay soil contaminated with 2.5% v/w crude oil soil with addition of zeolite incubated at 30 °C for a period of 30 days. Error bar represents SD of replicated experiments (n=3)

5.4.2 Influence of zeolite (clinoptilolite) and bacteria on the oil removal pattern in sandy soils

The sandy soil sample with and without addition of zeolite contaminated with the crude oil petroleum hydrocarbon had an initial bacterial mean population of 2.48 x 10^7 cfu/g and 2.39 x 10^7 cfu/g respectively **(Figure 5.9)**. The bacterial population in both (with and without zeolite) sandy soil treatments experienced a lag phase during the first 7 days but there was oil removal (15.1% and 10.3% respectively) during the period. The bacterial population subsequently gradually increased with no significant difference in the oil removal between

both sandy soil treatments during this period. The sandy soil with the zeolite had 79.0% ± 1.6% oil remaining while that without zeolite had 84.7% ± 1.6% remaining respectively. At day 14, the increase in bacterial cell population was maintained and decrease in oil level in both sandy soil amendments was also observed, with significant difference between the sandy soils with and without zeolite (60.2% ± 1.0% and 69.5% ± 1.2%, respectively). Interestingly, the sandy soil without the zeolite showed slightly more bacterial growth during the period than the sandy soil with zeolite but there was no difference in their cell population. At day 21, the oil level still continued to decrease in both soil treatments with significant differences in the oil removal, which was confirmed by the statistical analysis (< 0.0001). At day 30, the sandy soil with and without zeolite showed a significant difference in the oil removal, with 32.4% ± 0.9% and 40.3% ± 0.5% oil remaining respectively. Also at day 30, sandy soil spiked with crude oil amendments (control) with addition of zeolite but without bacterial consortium had oil remaining of 74.87% ± 1.17% while the crude oil spiked sandy soil without both treatments had 82.30% ± 1.47% oil remaining. For the control soil sample with only bacterial consortium, the cell population size decreased from the time of inoculation and at every sampling time below the inoculum size.

A comparative statistical analysis of the level significance in terms of the level of oil degradation in the clay and sandy soil treatment (S+C+B) during the period of investigation at each sampled time was compared **(Appendix III-Table III.4)**. It showed that T= 0, 7 and 14 day, there were no significant difference in the % oil removal between the bacterial consortium in the clay and sandy soils but that changed at day 21 and 30 where there were significant differences in the level of oil removal ($p< 0.0001$). For the zeolite-amended soils (S+C+B+Z), there were significant difference ($p< 0.0001$) between the oil removal level by the bacterial consortium in the clay and sandy soil at every sampled time during the period **(Appendix III-Table III.5)**.

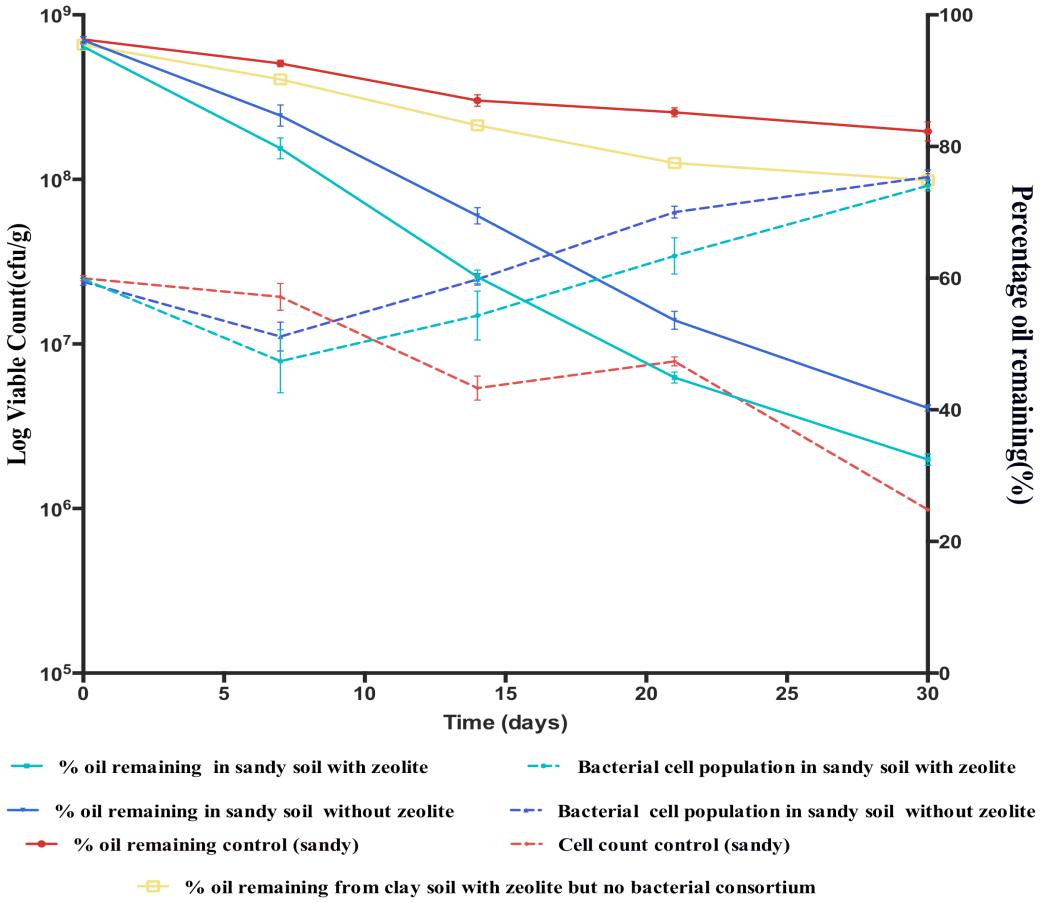

Figure 5.9 Bacterial growth and oil removal pattern in a sandy soil contaminated with 2.5% w/v crude oil soil with addition of zeolite incubated at 30 °C for a period of 30 days. Error bar represents SD of replicated experiments (n=3)

However, a further comparism of the bacterial consortium population size and oil removal levels in and between clay and sandy soil treatments (S+C+B+Z and S+C+B) at the end of the investigation (T=30 day) were analysed. There were significant differences ($p < 0.0001$) in the bacterial population size of all the clay and sandy soil treatments except for Sandy soil (S+C+B) vs. Sandy soil (S+C+B+Z) treatment with no significant difference in terms of the bacterial population size **(Appendix III-Table III.2)**. The % oil removal in and between all the clay and sandy soil treatments (S+C+B+Z and S+C+B) showed significant differences ($p < 0.0001$) at the end of the investigation **(Appendix III-Table III.3)**. This indicates that the addition of zeolite to soils does enhance oil removal.

5.4.3 Gas chromatographic analysis

Quantitative analyses of the residual oil extracted from the clay soil **(Figure 5.10)** and sandy soil **(Figure 5.11)** without zeolite augmentation and clay and sandy soil with zeolite amendments **(Figure 5.12)** were conducted using gas chromatographic analysis of the hydrocarbon extracted with hexane. This was carried out to quantify the individual hydrocarbons present in the petroleum crude oil. The GC analyses identified aliphatic compounds with carbon numbers between C_{10}-C_{28} **(Figure 5.10 and 5.11)**. The reduction in the peak area and heights from the chromatograms from the various extracts is an indication of reduction in the concentrations of the extracts.

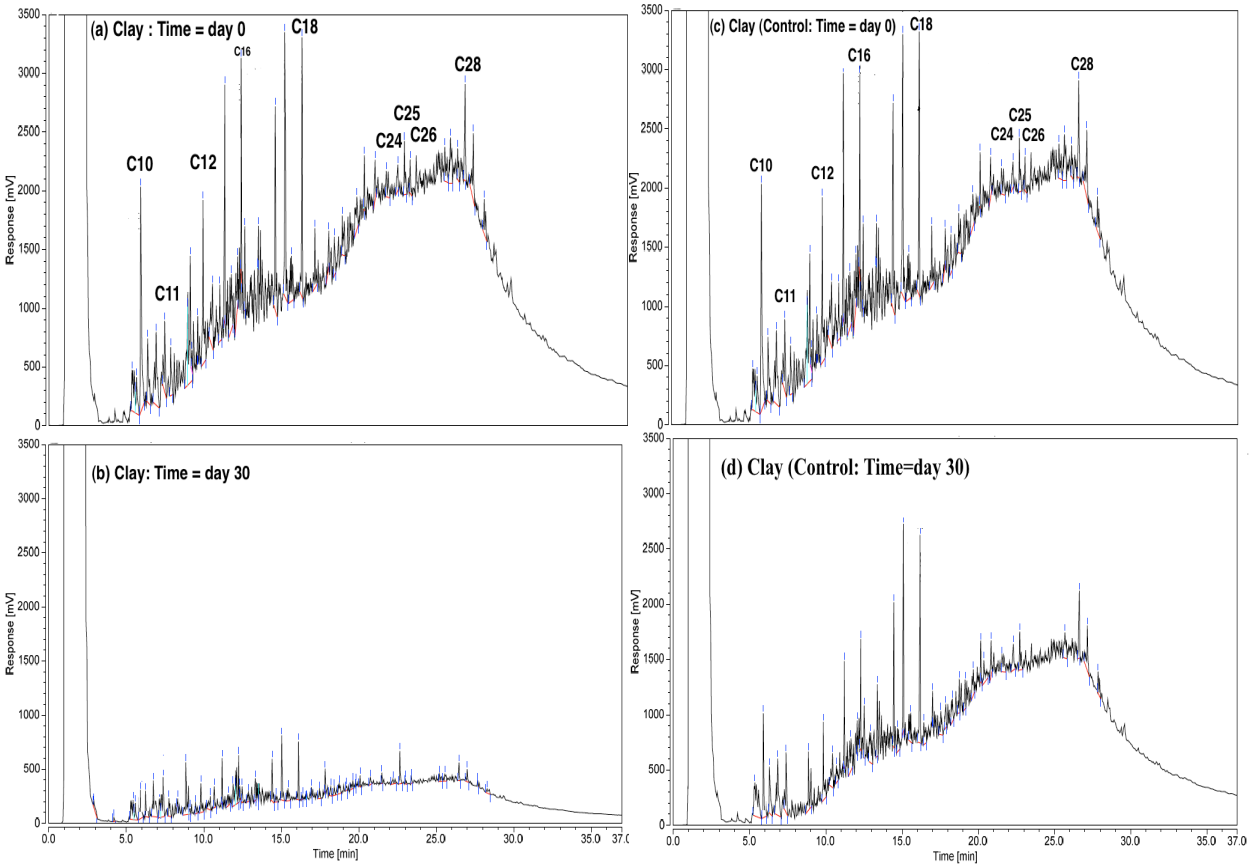

Figure 5.10 Gas chromatographic profiles of residual oil extracted from clay soil spiked with 1.5% (w/v) crude (a) with addition of bacterial consortium on day 0. (b) With bacterial consortium on day 30. (c) Without bacterial consortium on day 0 (d) without bacteria on day 30

The chromatograms of degraded oil recovered from the clay soil treatments at the start of the investigation (T=0) and the end day 30 and controls are presented in **Figure 5.10** while that of the sandy soil treatments and controls are presented in **Figure 5.11**. The reduction in the peak area and height from the residual oil extract from the clay soil where obviously lower than that of the sandy soil.

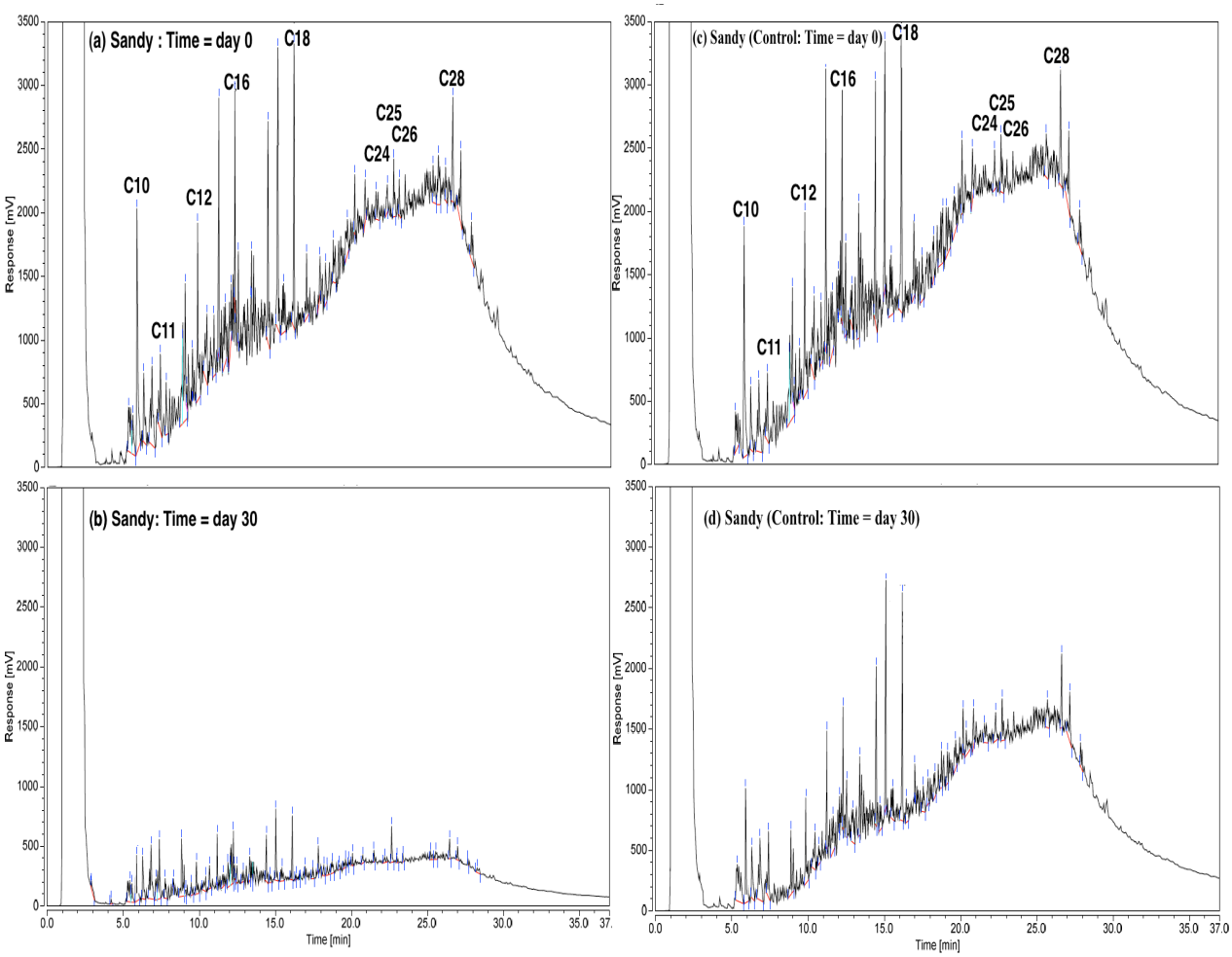

Figure 5.11 Gas chromatographic profiles of residual oil extracted from sandy soil spiked with 1.5% (w/v) crude (a) with addition of bacterial consortium on day 0. (b) With bacterial consortium on day 30. (c) without bacterial consortium on day 0 (d) without bacteria on day 30

The chromatograms of degraded oil recovered from silty-clay and sandy soil amended with zeolite at various time intervals are presented in **Figure 5:12**. It shows the trend of oil reduction based on decrease in peak area and height of the individual hydrocarbon.

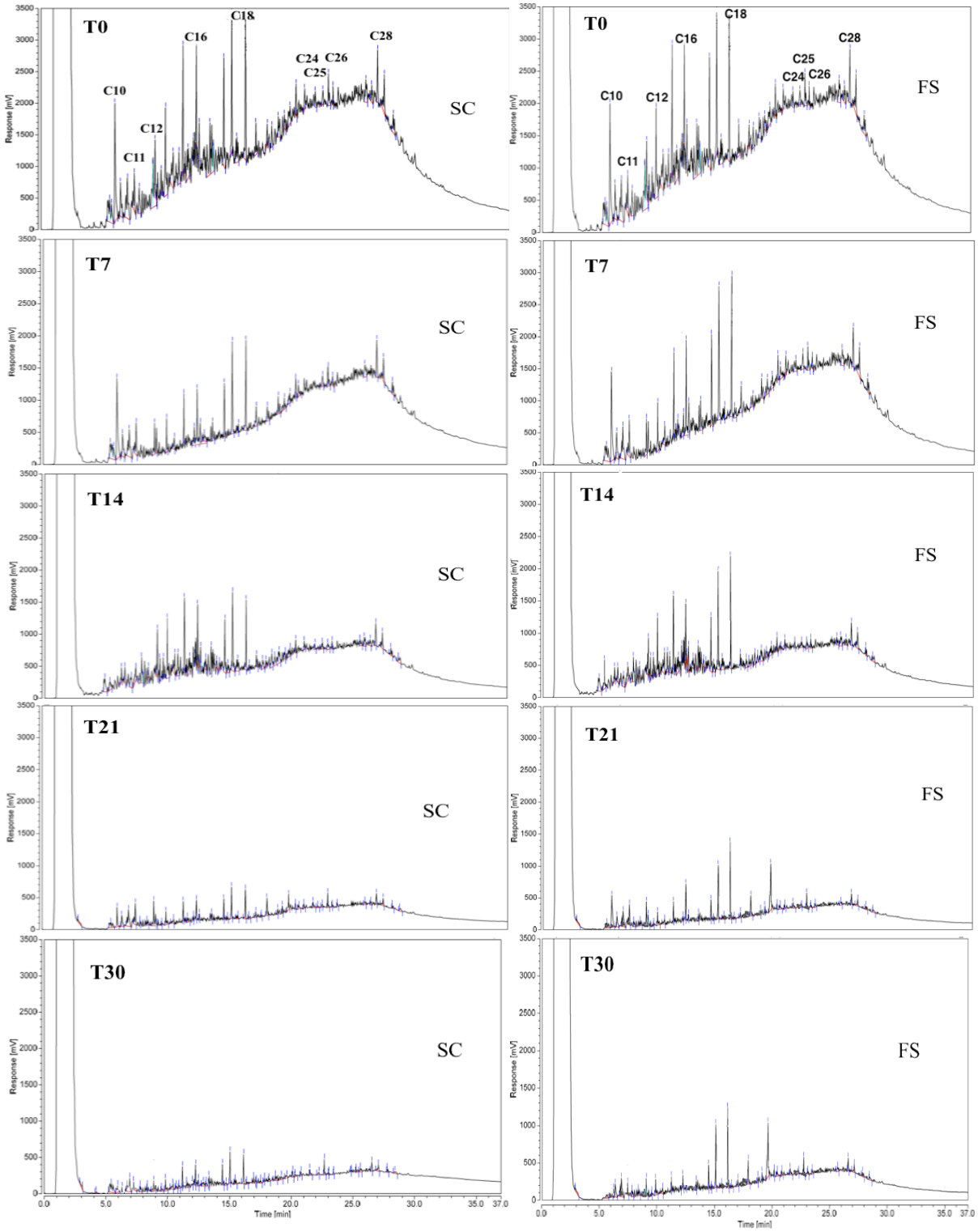

Figure 5.12 Chromatographic profiles of residual oil extract from the silty-clay (SC) and fine sand (FS) soils spiked with crude oil 2.5% (v/w) amended with zeolite (10 %w/w) and bacterial consortium after contamination (T0), 7 (T7), 14 (T14), 21 (T21) and 30 (T30) day

The peak areas from the hydrocarbon peaks were evaluated in relation to % removal of individual hydrocarbons. The quantitative analysis of the residual oil extracted from the silt-clay soil amendments (S+C+B+Z) and (S+C+B) at day 30 **(Figure 5.13)** revealed that C_{10}-C_{18} hydrocarbons were within the range of 92.1% - 57.7% and 80.4% - 44.8% degraded respectively. Hydrocarbon component (C_{24} and C_{25}) from S+C+B+Z were degraded to mean percentage of 39.4% ± 0.9% and 28.1% ± 0.7% respectively while S+C+B were 23.4% ± 0.8% and 19.0% ± 0.3% degraded. Hydrocarbons (C_{26}- C_{28}) from both treatments showed low degradation rates. Hydrocarbon components (C_{10}- C_{18}) from the quantitative analysis showed relatively high rates of degradation compared to (C_{24}- C_{28}). The control without bacterial consortium partially shows reduction in percentage of hydrocarbons C_{10}- C_{16} with no significant % reduction in C_{26}- C_{28} ($P > 0.0001$).

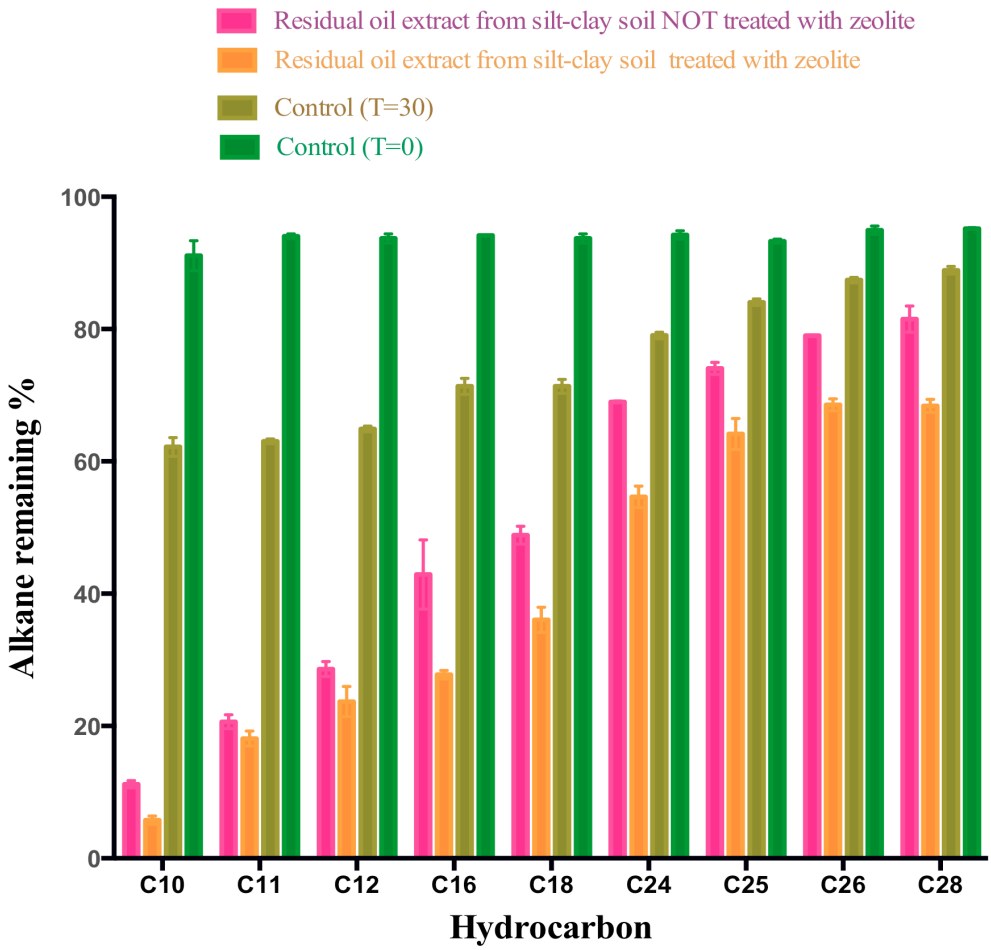

Figure 5.13 GC analyses of residual crude oil extract from silt-clay soil not treated with zeolite but with bacteria, silt-clay soil treated with zeolite and bacteria and control soil not treated with zeolite and bacteria (control) at day 0 and day 30. Error bar represents SD of replicated experiments (n=3)

Similarly, extracts from sandy soil amendments (S+C+B+Z) and (S+C+B) at day 30 **(Figure 5.14)** showed that hydrocarbons components C_{10}- C_{18} were within the range 74% - 43.7% and 69.4% - 42.8% degraded respectively. Hydrocarbon component (C_{24} and C_{25}) from S+C+B+Z were degraded to mean percentage of 25.1% ± 1.0% and 22.0% ± 0.7% respectively while S+C+B were 20.4% ± 0.8% and 16.0% ± 0.3% degraded. Hydrocarbons (C_{26}- C_{28}) from both treatments showed no significant degradation (P> 0.0001). The control without bacterial consortium partially shows reduction in percentage of hydrocarbons C_{10}- C_{18} with no significant % reduction in C_{26}- C_{28}. Hydrocarbon components (C_{10}- C_{18}) from the quantitative analysis showed relatively high rates of degradation compared to (C_{24}- C_{28}) (P> 0.0001).

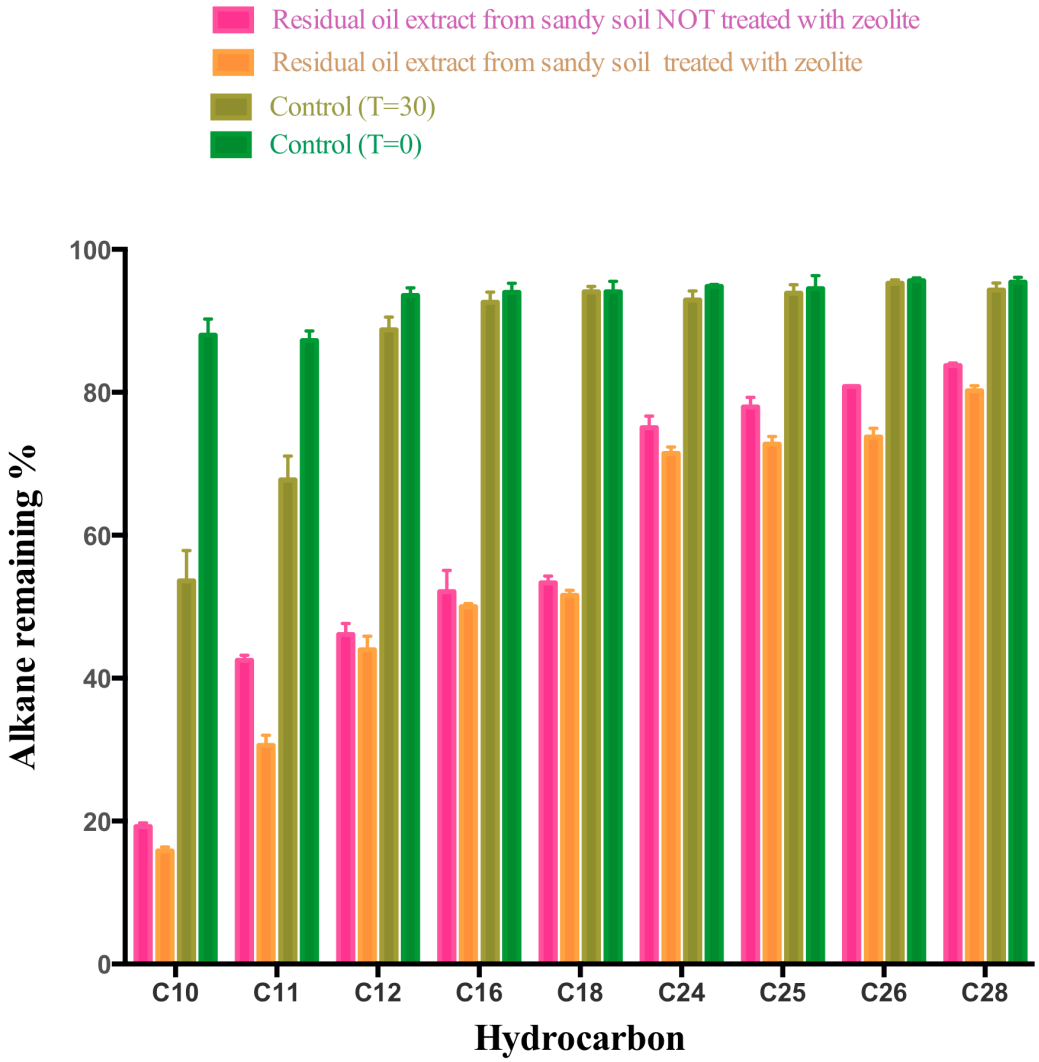

Figure 5.14 GC analyses of residual crude oil extract from fine sand soil not treated with zeolite but with bacteria, fine sandy soil treated with zeolite and bacteria and control soil not treated with zeolite and bacteria (control) at day 0 and day 30. Error bar represents SD of replicated experiments (n=3)

5.5 DISCUSSIONS:

This laboratory investigation was conducted on the modelled Niger Delta soil based on their particle sizes. A preliminary investigation was first conducted on the soil using zeolite and bacterial consortium (*Pseudomonas sp and Rhodococcus sp*) to provide an understanding of the remediation processes and a further investigation on the influence of soil particle sizes on the modelled clay and sandy soil of the Niger Delta region using the zeolite and bacterial consortium (*Pseudomonas aeruginosa, Rhodococcus sp, and Acinetobacter sp*). *Acinetobacter sp* was included to this investigation to enhance the bioremediation process due to its utilization in previous studies. The choice of the 30 °C temperature of incubation of the soil treatments is a reflection of the average temperature of the Niger Delta region (Effiong and Ayolagba, 2010).

These bacterial consortia used in the preliminary and main investigations had earlier been identified **(Chapter 3)** as capable of utilization of individual hydrocarbons as their sole carbon for growth and energy. Previous studies (Abdel-Megeed *et al*., 2010) have utilized *Pseudomonas spp* in bioremediation of phenolic compounds in bioremediation of petrochemical waste -waters (Ojumu *et al.,* 2004). A few studies have reported on the role of *Rhodococcus spp* in crude oil bioremediation. However, this author has not identified any studies utilizing a mixture of organisms with zeolite in the remediation of the soil environment of crude oil contaminants.

In the preliminary investigation, the lack of apparent growth during the first 14 days by the bacterial consortium **(Figure 5.3 & 5.5)** in both soil amendments with and without zeolite could be attributed to the adaptation period to the soil environment. However, the earlier soil analysis **(Chapter 4)** showed that the available nitrogen/phosphorus in the soil was probably too low to support bacterial growth. Thomas *et al.*, (1992) suggested that ratio of C: N: P needed to be able to sustain microbial activity should be 120:10:1. It was observed that the

total hydrocarbon to nitrogen and phosphorus in the soil samples used during this investigation was below the suggested ratio. Consequently, nutrient addition in the form of BSM on the 14th day **(Figure 5.5)** helped overcome this critical rate-limiting factor for the bacterial consortium in the contaminated soil environment. Also during this period, the bacteria did not increase in population size but the oil contents of the soil decreased substantially only in those soil treatments containing these non-growing bacteria would suggest that despite the bacteria not increasing in population size (partly due to lack of nitrogen or phosphorus) they still had the ability to metabolise hydrocarbons. However, this observation prompted the need to slightly modify the experimental design of the influence of soil structure on bioremediation experiment **(Section 5.2.6)** by using sterile BSM instead of sterile distilled to compensate for the moisture loss. The soils treatments with addition of zeolite had a higher bacteria population size at the end of the period of investigation **(Figure 5.3 & 5.8)**. This could be attributed to better aeration as a result of the size of the zeolite added to the soil which could have enhanced aeration (movement of oxygen) in the soil thereby increased the interfacial surface area and subsequently resulted to a higher degradation (Sharma and Pant, 2001). The zeolite particles are considered to be excellent carriers of bacteria, which enhance sludge activity in wastewater treatments. However, formation of bacterial layers on the zeolite surface is a slow process that could last for almost a week (Misaelide, 2011). The zeolite-clinoptilolite has been reported as a source of slowly release of potassium and nitrogen to the environment when needed and also act as water moderator, in which they can absorb up to 55% of the weight in water and slowly release it under the demand of plant and the ecosystem (Mumpton, 1999). This could probably justify why the zeolite treated soil had a higher bacterial population size during the period of the investigations.

At day 30 of the preliminary investigation the soil treated with zeolite had removed 79% of the oil while the un-treated soil with zeolite removed 67%. Soil amendment experiments showed more rapid and greater apparent oil removal with addition of bacterial consortium and clinoptilolite **(Figure 5.4 & 5.5)**. Similar results were also observed when the modelled clay and sandy amended with and without zeolite were experimented **(Figure 5.8 & 5.9)**. Oil removal was also observed in the soil, which was not treated with bacterial consortium but with zeolite (34%) and without both bacterial consortium and zeolite (16%). This removal could be attributed to abiotic factors either by volatilization or adsorption onto the particulate material. The pore diameters of zeolite are close to the critical dimensions of many hydrocarbon molecules there by allowing absorption of the molecules (Song *et al*., 2007). The soil environment determines the fate of PAHs in the environment. Organic contaminants may be accumulated within the soil the soil biota or be retained within the soil's mineral organic matter fractions while others may be lost by volatilization or biodegradation (Reid *et al*., 2000). This could have been responsible for the loss of the hydrocarbon contaminants from the control experiments. The fate of polluting oil in soil also depends on the amount of the oil spills' and the time of exposure (Ijah and Antai, 2003).

The quantitative analysis of the residual oil extracts during the preliminary investigation **(Figure 5.7)** shows greater reduction of C_{12} and C_{16} as compared to C_{26}-C_{28}. The hydrocarbon components C_{12} and C_{16} were degraded by bacterial consortium to 97% and 92% respectively from the zeolite amended soil while the soil not amended with zeolite had 78% and 88% removal. However, hydrocarbon components C_{24} - C_{28} where degraded by bacterial consortium to percentage mean of 36% and 29% respectively from the case of extracts amended with zeolite while without zeolite showed degradation of 33% and 27% respectively. However, further quantitative analysis of residual oil extract from the silt-clay and sandy soil amended with and without zeolite **(Figure 5.13 & 5.14)** clearly showed a high rate of

degradation for lighter hydrocarbon components (C_{10}– C_{18}) compared to the heavier ones (C_{24} – C_{28}) by the bacterial consortium. Hydrocarbon components (C_{10}– C_{18}) from both silty-clay and sandy soils amended with zeolite were degraded by the bacterial consortium to 92.1% - 57.7% and 74% - 43.7% respectively, while the soils without zeolite showed degradation rate of 80.4% - 44.8% (silt-clay) and 69.4% - 42.8% (sandy). Hydrocarbon components (C_{24}– C_{28}) from both soils showed an apparent low rate of degradation **(Figure 5.13 & 5.14)**. The observation that C_{10} – C_{18} hydrocarbons were easily and highly degraded than the C_{24} - C_{28} further justifies the findings of Atlas and Barth, 1981 which explain that smaller carbon chains are promptly degraded before the more complex hydrocarbon. The biodegradation of petroleum crude oil components usually occurs in the following order: alkanes, branched alkanes, the aromatic compounds and cycloalkanes (Antai and Mgbomo, 1993). Alkanes of intermediate chain length (C_{10}-C_{24}) are degraded more rapidly while very long chain alkanes are increasingly resistant to microbial degradation (Atlas and Barth, 1981). The control experiments of oil-contaminated soil without bacteria and zeolite amendments showed partial the lower hydrocarbon with C_{12} – C_{16}. This oil removal could be attributed to abiotic factors either volatilization or adsorption onto particulate material. Zeolite is reported to remove contaminants by the process of sorption or retardation (Carmody *et al*., 2007).

The fact that the different hydrocarbons in the residual oil were degraded at varying rates suggests that the bacterial consortium in the soil treatments utilized these hydrocarbons as preferential sources of carbon and energy. Also the addition of zeolite in the soil had enhanced the oil removal rate as demonstrated in Figure 5.7, as further justified in the quantitative analysis of the individual hydrocarbon components from both the silt-clay and sandy amended with zeolite **(Figure 5.13 & 5.14)**. The silt-clay amended with zeolite showed a greater oil removal rate compared to the sandy soil. This also agreed with the findings of Song *et al*., 2007 who reported that clays and zeolite exhibits more effective adsorbents for a

number of organic contaminants. Leggo *et al*., (2006) utilized natural zeolite and their modified forms in the reduction of heavy metals and hazardous substances. Zeolites are widely used in the decontamination of the environment of polluted soils (Coppola *et al*., 2003). Synthesized zeolite has also been reported to be used for photo-catalytic degradation of organic pollutants (Xamena *et al.,* 2003). The high cation exchange capacity of zeolite has helped in the removal of toxic metals (Gennaro *et al,* 2004) whereas bacteria remove contaminants such as oil hydrocarbon by degradation Okoh, (2006). The schematic illustrated shown in (**figure 5.13)** explains what happens to the contaminants molecules when they come in contact with zeolite and bacterial consortium.

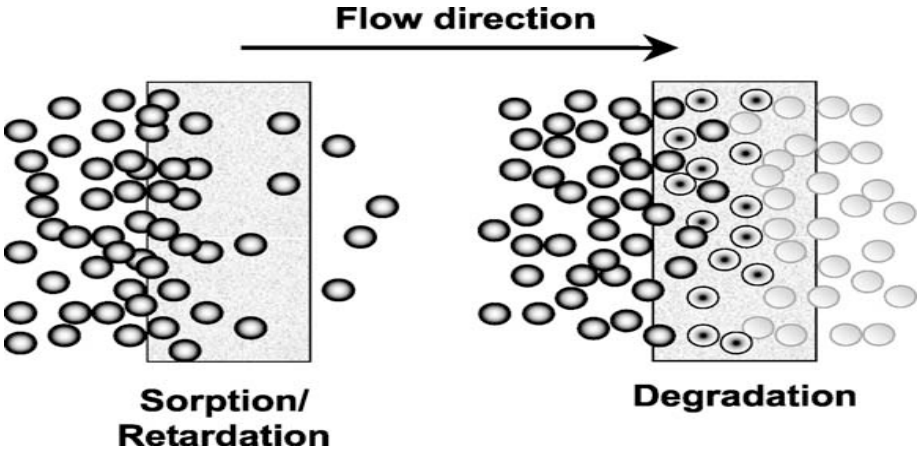

Figure 5.13 schematic illustration of movement of contaminants molecule in contact with zeolite and bacteria consortium

(**Source:** Bowman, (2003), *Microporous and Materials*)

Zeolite has the ability to contain contaminant e.g. oil spills preventing them from further pollution on land while the bacterial consortium gradually carry out the degradation process. The sorption reaction of contaminates is relatively fast but eventually the maximum capacity of the medium will be reached which was also observed in this investigation **(Figure 5.)** while the degradation of the contaminant by the bacterial consortium to a non-toxic compound is relatively slow (Bowman, 2002). The clay soil amendment zeolite (S+C+B+Z) showed a more rapid and greater extent and apparent oil removal by the bacterial consortium than the sandy soil amendments. They had an oil removal of 83.7% ± 0.6% and 62.7% ± 0.7% from

the zeolite amended clay and sandy soil respectively. The granular nature of the zeolite used would have opened up the pore of the clay soil and provided better aeration of O_2 for the bacterial growth thus enhanced the it's oil removal rates.

The soil type plays an important role in the fate of petroleum crude oil contaminant (Stemple *et al*., 2003). Bioremediation of contaminated silt-clay and sandy soils (S+C+B) had 72.6% ± 0.6% and 55.9% ± 0.6% oil removal respectively at the end of the period of the investigation. The oil removal in the silt-clay soil was significantly greater than that of the sandy soil. The effective adsorbents nature of clay for a number of organic contaminants as compared to sandy soil would have been responsible for its efficiency Song *et al*. (2007). Clay has been reported to increase the rate of oil breakdown by bacterial digestion (Chaerum and Tazaki, 2003). Also the colloidal size of clays has been reported to aid dispersion in hydrocarbon-polluted environment (Meyer and Quinn, 1973; Owen and Lee, 2003). In addition, the low organic carbon of the sandy soil used in this investigation (Table 5.2) would have been responsible for its lower degradation rates as a result of lower bacterial population size as compared to the clay soil with a higher organic carbon. This further justifies previous authors findings that soils with sandy texture and low organic carbon results in poor microbial proliferation and diversity and so bring about lower degradation rates to clay soil (Tally *et al*, 2002; Hejazi *et al*, 2004)

Hydrocarbon breakdown is influenced by the soil environment, soil area, composition of surface fractions and reactivity (Scherr *et al.,* 2007). The soil particle surfaces characteristic of clay and sandy particles used in this study was observed to play an important role in determine the distribution of hydrocarbon. The coarse particles of the sandy soil generally exhibit weak binding affinities of petroleum crude oil than the smaller particles of the clay as a result of decrease of surface area of larger particles. The clay has a non-homogeneous surface, which provides more sorption position for hydrocarbons and organic matter (Mott *et*

al, 1990). The high level of oil removal experienced in the amended clay soil as compared with the sandy soil could be associated with the increased bioavailability of this petroleum crude oil.

5.6 INTERIM CONCLUSIONS:

It is concluded that:

- The bacterial consortia of (*Pseudomonas sp + Rhodococcus sp) and (Pseudomonas aeruginosa, Rhodococcus sp, and Acinetobacter sp)* can bioremediate oil contaminated soil.

- Soil particle size composition significantly influenced the bacterial population size and oil removal from contaminated silt-clay and sandy soil composition

- The addition of zeolites (clinoptilolite) significantly enhanced bacterial oil removal in the contaminated silt-clay and sandy soil. The oil-contaminated clay soil augmentation with zeolite had a better oil removal rates than the sandy soil treated with zeolite.

- Biodegradation in a clay-contaminated soil showed a more efficient oil removal and bacterial population increase than in the sandy soil environment.

- The lighter hydrocarbon components (C_{10}– C_{18}) were degraded more rapidly compared to the heavier ones (C_{24} – C_{28}) by the bacterial consortium (in both the silt-clay and sandy soil amended with and without zeolite).

CHAPTER 6

6.1 GENERAL DISCUSSION

The three screened bacterial isolates (*Pseudomonas aeruginosa, Acinetobacter sp* and *Rhodococcus sp*) for ability to grow on petroleum hydrocarbon and at varying temperature was key to this investigation. These bacterial isolates have been shown from previous studies to survive the Niger Delta environment (Ayotamuna *et al.*, 2006). Identifying the warmest optimal temperature (30 ^{o}C) for the screened bacterial growth was also paramount to this investigation because it provided an understanding of the bacterial behaviour in a warm temperature and modelled environment of the Niger Delta. The ambient environment in the Niger Delta has a daily mean minimum and maximum temperature of 23 ^{o}C and 31.5 ^{o}C respectively (Ayotamuna *et al.*, 2006). The growth of these bacteria was observed to have been influenced by hydrocarbon concentrations with regards to the study on hexadecane. Aeration and agitation was observed to impact the level of bacterial growth and thus influenced bioremediation of petroleum hydrocarbon. This reflected in the methods employed in the growth of the bacteria, which influenced the choice of the shake flask method over the Bioscreen C Growth Analyzer.

In other to evaluate bacterial oil bioremediation in soil environments and the difficulty in obtaining soil samples from the Niger Delta region, it was necessary to model the soil in the laboratory. A thorough literature search of the Niger Delta soils (Iwegbue *et al*., 2006; Akpokodje, 1987) and Hilton soil (Fullen *et al*., 2006), the Hilton soil from East Shropshire, UK was used to model the Niger Delta soil because of their similar characteristic based on the XRF and XRD analyses **(Chapter 4)**. The modelled sandy and silty-clay used in this investigation were formulated based on their particle sizes composition were chosen based on that the two soil types are the most predominant soils in the Niger Delta region (Akpokdje, 1987).

The preliminary investigation on the modelled soil using the bacterial consortium (*Pseudomonas sp and Rhodococcus sp*) and established that nitrogen/phosphorus in the soil was low to support bacterial growth, thus necessitated nutrient addition. However, further investigation on the effects of soils structures on bioremediation was conducted on the two modelled Niger Delta soil particle size composition (silt-clay and sandy) using the bacterial consortium (*Pseudomonas aeruginosa, Rhodococcus sp, and Acinetobacter sp)* and established that soil particle size composition significantly influenced the bacterial population size and oil removal in contaminated clay and sandy soil composition. The addition of zeolites significantly enhanced bacterial oil removal in both the modelled soil types. It was observed that, biodegradation in a silt-clay-contaminated soil showed a more efficient oil removal and bacterial population increase than in the sandy soil environment and that the addition of zeolites significantly enhanced bacterial oil removal in the contaminated clay and sandy soil. In addition, oil-contaminated clay soil augmentation with zeolite had a better oil removal rates than the sandy soil treated with zeolite. Oil was removed either by evaporation, volatilization or photo-oxidation without any form of bacterial and/or zeolite treatments. Quantitative analysis of the hydrocarbon components showed that the lighter hydrocarbon components (C_{10}– C_{18}) were degraded more rapidly compared to the heavier ones (C_{24} – C_{28}) by the bacterial consortium (in both the silt-clay and sandy soil treatments with and without zeolite). It also established that the hydrocarbon components from the residual oil extract from the silt-clay soil was more degraded as compared to the sandy soil. This result is in line with the findings of Atlas and Barth, 1981 that reported that alkanes of intermediate chain length (C_{10}-C_{24}) are degraded more rapidly while very long chain alkanes are increasingly resistant to microbial degradation.

An engineered bioremediation strategy from the observations and conclusion of this investigation could help achieve the accessed oil polluted agricultural lands in the Niger

Delta. This could be carried-out *in situ* by first accessing and evaluating the site of contamination to identify the nature and extent of contamination. Crude oil is composed of more than 10,000 hydrocarbons and other molecule (Terry, 2014), evaluating the oil contaminated site will help provide an understanding of what is to be done and how it should be carried-out. A laboratory assay of the microorganisms from the site sample will be carried-out to identify microbes from the site with the potential to transform the contaminate. This would be optimized introducing growth –stimulating materials such as nutrients, oxygen through tilling the sites using machines e.g. ploughed and zeolite could be added and further tilled. The soil tillage would enhance aeration and the site can be optimized by spraying the soil with mineral nutrients to enhance the indigenous bacterial flora before introducing the bacterial consortium on the site to enhance the bioremediation. The agitation of the soil makes more oxygen available to the microorganism to grow and encourage biodegradation. Rhykerd *et al*., (1997) evaluated the impact of tillage, forced aeration and bulking agent on oil contaminated soil from an oil production site in east Texas. They reported that tilling of soil increased the rate and extent of remediation more than soil receiving forced aeration. At week 12 of their investigation, the total petroleum hydrocarbon content in the treatment with the tilled soil decreased by 82% whereas same treatment without tilling decreased by 33%. Tillage has been reported to enhance remediation of soils contaminated with tetrachloroethylene, trichloroethylene and 1,1,1-trichloroethane (Kempton *et al*., 1992).

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Addition of nutrient is also essential because oil pollution results in an imbalance in the carbon-nitrogen ratio at the site of the contamination, because crude oil is basically a mixture of carbon and hydrogen. A nitrogen deficiency could result in such a site, which may retard the growth of bacteria. It may also promote evaporation as well as loss of volatile and other petroleum components. The bacterial consortium could be introduced in the liquid form or in the powdered form and sprayed onto the soil. A soil tillage rate of 2-4 should be maintained (Ayotamuna *et al.*, 2006). For sites with oil spills, stocked bags of zeolite could be use to contain the flow of the oil preventing further contamination before the introduction of the bacterial consortium on it Bowman, (2003). Zeolites have been reported to be widely used in the decontamination of the environment of polluted soils (Oste *at al*., 2002; Coppola *et al*., 2003).

Guerin, (2000) carried out an investigation on soil from a former creosoting plant containing phenols and polycyclic aromatic hydrocarbons and was able to remediate the contaminated soil using a bioremediation processes involving soil mixing, aeration and addition of fertilizer. At the end of the investigation the 290 mg/kg PAH and 40mg/kg of phenol was reduced to <200 mg/kg and 2 mg/kg respectively. (Ayotamuna *et al.*, 2006) utilized a combination of treatments consisting of the application of fertilizers and oxygen exposure on a Niger Delta agricultural-soil contaminated with crude oil. At the end of the six weeks period of evaluation, it was reported that there was a reduction by 95% of the total hydrocarbon content as a result of nutrient addition.

6.2 GENERAL CONCLUSIONS

This understanding of the biodegradability potential of bacteria and zeolite addition in modelled soils was very important for recommending and developing an effective but

affordable bioremediation strategy for cleaning up oil spills that challenge communities in the Niger Delta, Nigeria (Diplock *et al.* 2009). Zeolite (clinoptilolite) was observed to enhance oil removal during the investigation possibly a consequence of its ability to contain oil spill by the process of sorption or retardation from further pollution (Carmody *et al*., 2007; Song *et at*., 2007). In addition, the granular nature of the zeolite used may have helped to open up the pore of the clay soil and provided better aeration of O_2 for the bacterial growth thus enhancing the it's oil removal rate. The soil type played an important role in the fate of petroleum crude oil contaminant, which was observed from the quantitative analysis of the residual hydrocarbon components extracts from both the silt-clay and sandy soil (Stemple *et al*., 2003). The lighter hydrocarbon components (C_{10}– C_{18}) were degraded more rapidly compared to the heavier ones (C_{24} – C_{28}) by the bacterial consortium (in both the silt-clay and sandy soil amended with and without zeolite).

The complexity of the Niger Delta region in terms of the vegetation, wetlands, creeks, coastal barrier sandy ridge, mangrove swamp, freshwater swamp, and lowland rainforest and small islands and how to access the oil spillages sites of could pose a challenge to the effective implementation of the bioremediation strategy. Oil spills in the region has been reported in very difficult areas such as creeks, vegetation and accessing the contaminated site is key to the success of the investigation (Francis *et al.,* 2011).

Conclusively, simply showing that these bacteria grown in the laboratory have the ability to degrade hydrocarbon contaminants is not enough but does provide an understanding of their growth and survival in the presence of oil-containing soils. What happens most especially *in situ* or under oil contaminated site conditions is very important to the success of the bioremediation. Oil spills have significant impact on vegetation, water supply, and marine life and local livelihoods dependent on agriculture (EIA 2003). The people's predominant occupation in the Niger Delta region is agriculture and fishing (Francis *et al.,* 2011). To be

able to reclaim back some of the oil polluted lands for agricultural purposes could greatly enhance livelihood and the ecological state of the region. This research investigation has laid the fundamental foundation for further *in situ* bioremediation application of the recommended strategies in the Niger Delta region.

6.3 RECOMMENDATIONS

The following recommendations from the investigation are suggested as a strategy for effective bioremediation in the Niger Delta region:

- Bioremediation should be applied during the dry season because of the wet nature of the Niger Delta region. In other to enhance the tillage of the soil and to improve the soil aeration because wet soil induces anaerobic conditions that could delay bioremediation processes
- Stock bags of zeolite (Clinoptilolite) should be used as a barrier in the Niger Delta region to contain oil spills, which could prevent further contamination of oil from flowing into the streams, rivers.
- Application of zeolite to an oil contaminated soil site and soil tillage method should be employed to achieve an accelerated biodegradation. A granular nature (pellet) of zeolite can be used to help open up the pore of the silt clay soil and provided better aeration of O_2 for the bacterial growth thus enhanced the it's oil removal rates
- Nutrient like sterile BSM or fertilizers should be used to biostimulate the oil contaminated soil of indigenous microbial flora and engineered bacterial consortium could be applied on to the site in powder form or liquid form by sparing to enhance biodegradation.

APPENDIX 1

Table I. 1 Screened bacteria for hydrocarbon degradation for a period of 7 days at varying temperatures using the plate methods

Bacteria code/ Names	25ºC			30ºC			37ºC			40ºC			50ºC		
	TSA	HEX	SB	TSA	HEX	SB	TSA	HEX	SB	TSA	HEX	SB	TSA	HEX	SB
575	+	+	-	+	-	-	+	-	-	+	-	-	-	-	-
576	+	+	-	+	-	-	+	-	-	+	-	-	-	-	-
405	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-
401	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
238	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-
413	+	+	-	+	+	-	+	+	-	+	-	-	-	-	-
545	+	+	-	+	+	-	+	+	-	+	-	-	-	-	-
546	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-
405	+	+	+	+	-	-	+		-	+	-	-	-	-	-
476	+	+	+	+	-	-	+	+	-	+	-	-	-	-	-
475	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
GP	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Rhodococcus sp	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Pseudomonas sp	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Bacillus sp	+	-	+	+	+	-	+	-	-	+	-	-	-	-	-
Bacillus sp	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Enterobacter sakasaki	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Acinetobacter sp	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Geobacillus sterothermophilus	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-
Bacillus sterothermophilus	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-

Media was incubated in plates (+) positive growth and (-) negative grown

Table I. 2 shows the preparation of solution of 10ml BBM+ hexadecane to be used during the Bioscreen experiment

Final Percentage concentration of hydrocarbon expected % (v/v)	Equivalent Volume of inoculum in 10mls solution (ml)	Volume of BBM Solution BBM = (10ml – Inoculum - Hydrocarbon) (ml)	Volume of hydrocarbon (ml)
0.0	0.67	9.33	0.00
0.5	0.67	9.28	0.05
1.0	0.67	9.23	0.10
2.0	0.67	9.13	0.20

Table I. 3 shows the volume of each solution to be pipetted into the plate well in line with the specific hydrocarbon concentration

Percentage concentration of hydrocarbon % (v/v)	Volume of inoculum used (µl)	Volume of BBM Solution (µl)
0.0	20	280
0.5	20	280
1.0	20	280
2.0	20	280

Growth Calculation and Analysis

The growth rate constant and mean generation time of the bacteria isolates where calculated using

Calculation I.1: Growth rate constant (μ) = $\frac{(\log_{10} OD_2 \log_{10} OD_1)\ (2.303)}{(t_2 - t_1)}$

Calculation I.2: Mean generation time (g) = $\frac{\text{In } 2}{\mu}$

Where OD is the optical density and t, time difference

Example I.1: Illustrate how the growth rate constant and mean generation time of *Pseudomonas aeruginosa* was determined **(from Figure 3.2)** as shown in **Table 3.2** in the main text.

Considering two points on the typical graphical representative (2.0% hexadecane) concentration **(see Figure 3.2)**

Parameters:

Optical density: OD_2: 0.38 and OD_1: 0.22

Time (hours): t_2: 30 h and t_1: 14 h

Growth rate constant (μ) = $\frac{(\text{Log}_{10}\ 0.36 - \text{Log}_{10}\ 0.22)\ (2.303)}{(30 - 14)}$

$= \frac{0.2139 \text{ X } 2.303}{16}$

(μ) = $0.031 h^{-1}$

Mean generation time (g) = $\frac{\text{In } 2}{\mu}$

$= \frac{0.693}{0.031}$

(g) = 22.353 h

APPENDIX II

Table II. 1a shows the mineralogical composition of the different particle sizes from the XRD analysis.

Particle sizes (mm)	Card ID	Match Score	Record mean Score	I (%)	Displ (μm)	Formula
< 0.063	05-0490 03-0418 03-0447 19-1227 41-1443	27.68 11.44 9.74 13.39 7.00	0.77 0.42 0.44 0.20 0.28	43 4 8 7 10	70 131 -51 -51 -42	SiO_2 Ca-Mg-Al-Si-O $AlPO_4$ $(K,Na)\ (Si_3Al)\ O_8$ MgF_2
0.063 – 0.125	03-0444 42-1478 03-0447 26-0316 41-1476	19.25 12.93 9.25 10.92 5.33	0.52 0.23 0.42 0.27 0.48	7 14 14 8 8	-99 62 12 30 -4	SiO_2 $Ca_4Al_60_{12}SO_4$ $AlPO_4$ $CaMnSi_4O_{10}$ KCl
0.125 – 0.250	03-0444 03-0447 42-1478 03-0452 36-1377	21.48 10.79 13.18 9.80 1.87	0.58 0.49 0.23 0.29 0.62	4 7 5 7 2	-34 91 8 -112 70	SiO_2 $AlPO_4$ $Ca_4Al_6O_{12}SO_4$ $FeP0_4.2H_20$ Mg0.9Mn0.10
0.250 – 0.500	03.0444 03.0447 36-1377 42.0429 03-1177	20.60 10.43 2.23 4.82 2.78	0.56 0.47 0.74 0.10 0.17	3 7 1 6 1	-37 12 20 -46 -17	SiO_2 $AlPO_4$ MgO.9Mn0.10 $Fe4(PO_4)3(OH)_3$ $AlMn_8.4$
0.500 – 1.000	03-0444 03-0447 26-0316 36-1377 23-0493	19.73 10.85 10.79 2.26 2.98	0.53 0.49 0.26 0.75 0.27	4 10 5 2 7	-75 12 -4 -26 40	SiO_2 $AlPO_4$ $CaMnSi_4O_{10}$ MgO.9MnO.10 K_2O
1.000 – 2.000	05-0490 03-0418 03-0447 36-1377 06-0695	29.85 13.77 10.14 2.36 3.62	0.83 0.51 0.46 0.79 0.33	43 3 7 2 2	73 131 -64 -125 -54	SiO_2 Ca-Mg-Al-Si-O $AlPO_4$ MgO.9MnO.10 Fe_3Al

Table II.1b Shows the physiochemical properties of the different Hilton soil particle sizes

Particle Size (mm)	Physiochemical properties			
	pH	Organic Carbon (%)	Nitrogen (%)	Available PO_4 (ppm)
< 0.063	6.73 ± 0.20	26.4 ± 0.3	0.17 ± 0.01	1.6 ± 0.2
0.063 – 0.125	6.70 ± 0.28	21.6 ± 0.1	0.16 ± 0.02	1.6 ± 0.2
0.125 – 0.250	6.63 ± 0.13	18.3 ± 0.2	0.13 ± 0.02	1.4 ± 0.1
0.250 – 0.500	6.67 ± 0.34	19.6 ± 0.4	0.13 ± 0.01	1.4 ± 0.3
0.500 – 1. 000	6.61 ± 0.19	17.3 ± 0.3	0.12 ± 0.03	1.1 ± 0.2
1.000 – 2.000	6.58 ± 0.22	16.1 ± 0.4	0.11 ± 0.02	1.1 ± 0.5

Results represent the mean±standard deviation of three replicates

Table II. 2 Shows the elemental concentration present in the modelled clay and sandy soil type

Element	Elemental concentration (%)	
	Clay	Sandy
Na	0.82	0.51
Mg	0.576	0.495
Al	4.456	3.263
Si	37.03	46.15
P	0.1016	0.0708
S	0.1519	0.1148
CI	0.01281	0.01205
K	1.297	0.865
Ca	0.4908	0.3162
Ti	0.2904	0.1158
V	0.00335	<0.0017
Cr	0.0075	0.00384
Mn	0.0456	0.0224
Fe	2.086	1.031
Co	<0.0027	<0.0019
Ni	0.00366	0.00341
Cu	0.00215	0.00112
Zn	0.00845	0.00298
Ga	0.00088	0.0006
Ge	0.00021	<0.00015
As	0.00085	0.0004
Se	0.00008	<0.00009
Br	0.00087	0.00027
Rb	0.00766	0.00519

Element	Elemental concentration (%)	
	Clay	Sandy
Sr	0.00595	0.00407
Y	0.00172	0.00092
Zr	<0.050	<0.050
Nb	<0.0011	<0.00074
Mo	<0.0030	<0.0037
Ag	<0.00069	<0.00060
Cd	<0.00088	<0.0011
In	<0.00083	<0.00071
Sn	<0.0019	<0.0018
Sb	<0.0011	<0.0011
Te	<0.0020	<0.0020
I	<0.0030	<0.0034
Cs	<0.0046	<0.0049
Ba	0.0357	0.0262
La	<0.0097	<0.0100
Ce	<0.013	<0.013
Hf	0.00052	0.00051
Ta	0.00381	0.00292
W	0.00248	<0.00056
Hg	<0.00028	0.00018
Ti	<0.00027	<0.00021
Pb	0.00895	0.00326
Bi	<0.00024	0.00016
Th	0.00077	0.00025
U	<0.00063	<0.00048

Table II. 3 Shows the elemental concentration of zeolite (clinoptilolite)

Element	Element Concentration %
Na	< 0.17
Mg	< 0.027
Al	0.1997
Si	5.906
P	< 0.00072
S	0.00571
Cl	0.06597
K	0.984
Ca	0.4327
Ti	0.0508
V	< 0.0011
Cr	0.00137
Mn	0.0125
Fe	0.9234
Co	< 0.0015
Ni	0.00226

Element	Element Concentration %
Cu	0.00080
Zn	0.00262
As	0.00019
Se	< 0.00008
Rb	0.01144
Sr	0.06314
Zr	< 0.050
Mo	< 0.0021
Cd	< 0.0014
Sn	< 0.0017
Sb	< 0.0011
Ba	0.0317
W	< 0.00042
Hg	< 0.00019
Pb	0.00065

APPENDIX III

Table III.1 Statistical analysis comparing residual oil extracted from various soil treatments. **(Preliminary investigation)**

Residual oil extract from soil treatments	**P-value**
Soil with zeolite + bacterial consortium / Soil without zeolite & bacterial consortium	0.0013
Soil with zeolite + bacterial consortium / Soil with zeolite &without bacterial consortium	< 0.0001
Soil without zeolite & bacterial consortium / Soil with zeolite & without bacterial consortium	< 0.0001
Soil with zeolite & without bacterial consortium / Soil without zeolite & without bacterial consortium	< 0.0001

Table III.2 statistical analyses comparing bacterial cell growth from the different soil treatments and types at day 30 of the investigation

Comparing the cell population from the different soil types and treatments	**Level of Significance**
Clay soil (S+C+B) vs. Sandy soil (S+C+B)	< 0.0001
Clay soil (S+C+B) vs. Clay soil (S+C+B+Z)	< 0.0001
Clay soil (S+C+B) vs. Sandy soil (S+C+B+Z)	< 0.0001
Sandy soil (S+C+B) vs. Clay soil (S+C+B+Z)	< 0.0001
Sandy soil (S+C+B) vs. Sandy soil (S+C+B+Z)	n/s
Clay soil (S+C+B+Z) vs. Sandy soil (S+C+B+Z)	< 0.0001

n/s stands for no significant difference in the % oil remaining comparing the various treatments in both clay And sandy soil

Table III.3 statistical analysis comparing percentage oil removals residual from the different soil treatments and types at day 30 of the investigation

Comparing the oil removal from the different soil types and treatments	**Level of Significance**
Clay soil (S+C+B) vs. Sandy soil (S+C+B)	< 0.0001
Clay soil (S+C+B) vs. Clay soil (S+C+B+Z)	< 0.0001
Clay soil (S+C+B) vs. Sandy soil (S+C+B+Z)	< 0.0001
Sandy soil (S+C+B) vs. Clay soil (S+C+B+Z)	< 0.0001
Sandy soil (S+C+B) vs. Sandy soil (S+C+B+Z)	< 0.0001
Clay soil (S+C+B+Z) vs. Sandy soil (S+C+B+Z)	< 0.0001

Table III.4 Compares the level of significance of the oil removal between the crude Oil- contaminated silt-clay and sandy soil (S+C+B) during the period of investigation.

Period of oil removal: Clay soil vs Sandy soil (Days)	**Level of Significance**
0	n/s
7	n/s
14	n/s
21	< 0.0001
30	< 0.0001

N/s stands for no significant difference in the % oil remaining comparing the various treatments in both silt-clay and sandy soil.

Table III.5 Compares the level of significance of the oil removal between the crude oil- contaminated Silt-clay and sand soil (S+C+B+Z) during the period of investigation.

Period of oil removal: Clay soil vs Sandy soil (Days)	Level of Significance
0	n/s
7	< 0.0001
14	< 0.0001
21	< 0.0001
30	< 0.0001

n/s stands for no significant difference in the % oil remaining comparing the various treatments in both silt-clay and sandy soil

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